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Purification of an Acetylcholine Receptor from a Nonfusing Muscle Cell Line†

Jim Boulter* and Jim Patrick

ABSTRACT: A nicotinic acetylcholine receptor has been solubilized and purified from the nonfusing, mouse muscle cell line BC_3H1. The presence of an acetylcholine receptor was monitored throughout the purification by the specific binding of α-[^{125}I]bungarotoxin. Affinity chromatography of Triton X-100 solubilized BC_3H1 membranes on an agarose-α-neurotoxin conjugate followed by sucrose density gradient centrifugation resulted in a 1700-fold purification of the α-[^{125}I]bungarotoxin binding component with a final specific activity of 2600 pmol of α-[^{125}I]bungarotoxin binding sites per mg of protein. The purified acetylcholine receptor has an apparent sedimentation coefficient of 9.5×10^{-13} s, is a glycoprotein containing glucosyl and/or mannosyl residues but no detectable D-fucose, D-galactose, or *N*-acetyl-D-glucosamine, and shares some antigenic determinants with acetylcholine receptors purified from *Electrophorus electricus* and *Torpedo californica*. Polyacrylamide gel electrophoresis of the purified acetylcholine receptor in the presence of sodium dodecyl sulfate revealed four subunits with apparent molecular weights of 72 000, 65 000, 53 000, and 44 000. A radioactive preparation of purified acetylcholine receptor has been obtained from BC_3H1 cells cultured in the presence of [^{3}H]leucine. Sucrose density gradient analysis of the [^{3}H]acetylcholine receptor demonstrates that all of the α-[^{125}I]bungarotoxin binding activity and 95% of the [^{3}H]leucine radioactivity migrate as a single symmetrical peak with an apparent sedimentation coefficient of 9.5×10^{-13} s. Evidence is presented which suggests that the purified acetylcholine receptor preparation contains a mixture of both "surface" and "hidden" receptor populations. Finally, a rabbit anti-BC_3H1 acetylcholine receptor antiserum has been prepared.

The acetylcholine receptor (AcChR)[1] has now been purified and characterized by a number of laboratories from a variety of sources (Miledi et al., 1971; Biesecker, 1973; Schmidt and Raftery, 1973; Chang, 1974; Lindstrom and Patrick, 1974; Meunier et al., 1974; Weill et al., 1974, Merlie et al., 1975; Brockes and Hall, 1975a). Although a vast literature concerning the physicochemical nature and general pharmacology of the AcChR from these sources is available, less is presently known concerning the cellular biochemistry of the AcChR. Recently, however, considerable progress has been made in this area utilizing organ and cell culture techniques (Brockes and

† From the Department of Neurobiology, The Salk Institute, San Diego, California 92112. *Received May 3, 1977.* J.B. was supported by National Institutes of Health Postdoctoral Fellowships 1 F22 NS00246 and 1 F32 NS05669. This work was supported by grants from the National Institutes of Health and the Muscular Dystrophy Associations of America, Inc., to J.P.

[1] Abbreviations used are: α-[^{125}I]BuTx, α-[^{125}I]bungarotoxin; α-NTx, α-neurotoxin; AcCh, acetylcholine; AcChR, acetylcholine receptor; PBS (phosphate-buffered saline), 138 mM NaCl, 3 mM KCl, 0.9 mM $CaCl_2$, 0.05 mM $MgCl_2$, 8.15 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2; 0.5% PBS, PBS containing 0.5% (w/v) Triton X-100; 3% PBS, PBS containing 3% (w/v) Triton X-100; dTC, *d*-tubocurarine; Prep medium, Dulbecco modified Eagle's medium in which the bicarbonate is replaced with 1.08 mM Na_2HPO_4–1.5 mM K_2HPO_4; BSA, bovine serum albumin; Na-$DodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Con A, concanavalin A.

Hall, 1975a, Merlie et al., 1975; Devreotes and Fambrough, 1975, 1976; Devreotes et al., 1977; Patrick et al., 1977). In terms of experimental design, such organ and cell culture systems afford unique opportunities for examining the synthesis, degradation, regulation, and topology of acetylcholine receptors.

Experiments in this laboratory (Patrick et al., 1977) have been directed toward an investigation of AcChR biochemistry in the clonal, mouse myogenic cell line BC_3H1. This cell line was isolated by Schubert et al. (1973) from a nitrosoethylurea-induced intracranial neoplasm in strain C_3H mice and has been kept in continuous cell culture for over 3 years. The BC_3H1 cells possess biochemical, morphological, ultrastructural, and electrical properties characteristic of vertebrate smooth muscle and as these cells reach confluency they cease dividing, become highly elongated while remaining mononucleate, and synthesize large amounts of a nicotinic acetylcholine receptor. (See Schubert et al. (1973) for a complete description of the properties of the BC_3H1 cell line.) These relatively high levels of AcChR (2–3 pmol of toxin binding sites per mg of cell protein), combined with the ability to culture gram quantities of the BC_3H1 cells, have facilitated the purification and initial characterization of this mammalian acetylcholine receptor.

In this report we present the purification and characterization of the AcChR from the BC_3H1 cell line as well as the preparation of a rabbit anti-BC_3H1 AcChR antiserum. Such antisera should prove extremely useful for studies on the topology and localization of the AcChR in the limiting cell membrane and as a general reagent for exploring fundamental aspects of acetylcholine receptor biochemistry in the BC_3H1 cell line.

Experimental Section

Materials and Methods

Media and Growth of Cells. The BC_3H1 cells were grown in Dulbecco modified Eagle's medium containing 20% fetal calf serum in an atmosphere of 88% air and 12% CO_2 at a temperature of 37 °C. We routinely used 1 mL of culture medium per 5.9 cm^2 of tissue culture dish surface area. The BC_3H1 cells grow as a monolayer with a generation time of 15–17 h. The cells were plated at an initial density of 1.8×10^3 cells per cm^2 and, at confluency, reached a final cell density of 7.1×10^4 cells per cm^2. Confluency was attained in about 5–7 days while maximum AcChR densities were achieved by 10–12 days. Cell passage was performed every 4 days and the cells were always passed in the exponential phase of growth. For large scale production of BC_3H1, the cells were plated on 150×25 mm plastic tissue culture dishes (Falcon Plastics) and harvested as follows: the medium was removed on day 12 and the cells washed once with 10 mL of Prep medium and once with 10 mL of PBS at 4 °C. The cells were removed from the dishes with a large rubber spatula and pelleted by centrifugation for 10 min at 500*g* in a Model HN-S centrifuge (International Equipment Co.). The cell pellet was washed once with 25–50 mL of PBS and centrifuged at 8000*g* for 10 min in a Sorvall RC-2B centrifuge. If the cells were not used immediately, the pellet was taken up in Prep medium containing 10% fetal calf serum (1 mL of medium per g wet weight of cells) and frozen in liquid nitrogen using a Biological Freezer type BF-5 (Union Carbide) at a cooling rate of 1 °C per minute. Frozen cells have been kept up to 2 months with less than a 10% loss in the total amount of α-[^{125}I]BuTx binding activity.

Preparation and Iodination of Snake Venom Neurotoxins. α-Neurotoxin (α-NTx) from the venom of the cobra *Naja naja Kaothia* (Biologicals Unlimited) and α-bungarotoxin (α-BuTx) from the venom of the krait *Bungarus multicinctus* (Ross Allen Serpentarium) were purified as described earlier (Patrick et al., 1972) and iodinated using the iodine monochloride method (Vogel et al., 1972). The mono and diiodo derivatives were separated by chromatography on CM-Sephadex (Pharmacia) and the diiodo toxin was used for all experiments. Iodinated α-BuTx had specific activities between 7×10^7 and 1.5×10^8 cpm per nmol.

Preparation of Affinity Columns. Protein coupling to cyanogen bromide activated agarose A-50m (Bio-Rad) was performed essentially as described by Cuatrecasas (1970). α-Neurotoxin was coupled to a final concentration of 0.1 mg of protein per mL of packed agarose and the conjugate stored at 4 °C in PBS. Just prior to use the α-NTx–agarose conjugate was washed with: (a) 10 vol of 2 M urea plus 0.5 M NaCl, (b) 10 vol of PBS containing 1 M NaCl, and (c) 20 vol of PBS containing 3% Triton X-100. The α-NTx–agarose conjugate was made fresh for each AcChR purification and was used within 72 h. Conjugates of agarose A-50m and plant lectins (at 1 mg of protein per mL of packed agarose) were made as above and stored at 4 °C in PBS plus 1 mM sodium azide until needed.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard. In extracts containing Triton X-100 an appropriate Triton X-100 containing blank was always used to correct for the positive Lowry reaction of the detergent. The precipitate observed upon addition of the Folin reagent was removed by centrifugation at 10 000*g* for 10 min prior to reading the absorbance at 700 nm.

Protein concentrations in the purified AcChR preparations were determined by a fluorescent protein assay (Bohlen et al., 1973) using fluorescamine (Fluram, Roche Industries) as the fluorescent reagent. Fluorescamine was used at 0.3 mg per mL in histochemical quality dioxane. The fluorescence was measured with a Hitachi Perkin-Elmer spectrofluorometer Model MPF-2A and a Hitachi QPD recorder. Excitation was at 390 nm (slit width 4 nm) and the fluorescence was monitored by continuous scanning between 410 and 500 nm (slit width 6 nm) with the maximum fluorescence at or near 475 nm. The relative fluorescence for the AcChR sample was determined at three different protein concentrations and corrected for reagent blanks. The reagent blanks were prepared exactly as for the AcChR protein determination except that the sample was obtained from a position in a blank sucrose gradient which corresponded to the position of the AcChR in the experimental gradients. The concentration of AcChR protein in the samples was obtained from a standard plot of relative fluorescence vs. protein concentration using dialyzed bovine serum albumin as a standard. The concentration of the stock BSA solution was determined spectrophotometrically using an extinction coefficient of $E_{280}^{0.1\%} = 0.67$.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed essentially as described by Weber and Osborn (1969). After electrophoresis, the protein was fixed in situ and the $NaDodSO_4$ removed by repeated washes of the gel in a solution of 50% (v/v) methanol and 10% (w/v) trichloroacetic acid. The staining of the gels with Coomassie blue and subsequent destaining were performed as described by Fairbanks et al. (1971).

Because the concentration of the purified AcChR was so low (approximately 2–5 μg per mL) a special procedure was developed to permit an adequate amount of protein to be applied

to the gel in the smallest volume possible. The SW 41 sucrose density gradient pooled AcChR (approximately 4–5 mL) was placed in a siliconized, screw-capped vial with a small stirring bar. To this was added 25 μL of packed DE-52 anion exchange resin preequilibrated with 0.01 M Tris, 0.01 M NaCl, and 0.2% Brij-35 (pH 8.0). This mixture was stirred gently in the cold for 30 min. At the end of this period, the DE-52 was pelleted by centrifugation at 50*g* and the supernatant was removed. Under these conditions greater than 95% of the α-[^{125}I]BuTx binding activity is adsorbed to the DE-52. The DE-52 was then taken up in 0.5 mL of the equilibration buffer and transferred to an Eppendorf microfuge tube and centrifuged at 12 000*g* for 2 min and the supernatant was removed by aspiration. To the packed DE-52 was added 100 μL of sample preparation buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid, 20 mM dithiothreitol, 2% (w/v) $NaDodSO_4S$, 5% (w/v) sucrose, and 0.002% (w/v) bromophenol blue, pH 8.0), and the slurry was heated at 100 °C for 3 min. The mixture was cooled and centrifuged at 12 000*g* for 2 min and the supernatant removed with a Hamilton syringe and loaded directly on the gel. It should be noted that although this procedure allows one to effectively concentrate the AcChR and at the same time remove most of the unwanted detergent from the sample, the procedure may not result in quantitative removal of the AcChR from the DE-52 resin. The stained gels were scanned at 560 nm with a Gilford model 2400S spectrophotometer equipped with a linear transport system and a 0.10 × 2.36 slit width filter.

Assay for Acetylcholine Receptor. Throughout the purification procedure the AcChR was assayed by determining the number of α-[^{125}I]BuTx binding sites present in a given sample. The details of the α-[^{125}I]BuTx binding assay to both whole BC_3H1 cells and solubilized BC_3H1 AcChR have been described by Patrick et al. (1977). With solubilized AcChR an aliquot of the sample was incubated with α-[^{125}I]BuTx (final concentration of 2×10^{-8} M) for 1 h at 4 °C. The sample was then applied to gradients of 5–20% sucrose in 0.5% PBS and sedimented in an SW 60 rotor at 45 000 rpm for 16 h at 2 °C. The gradients were fractionated with a Hoeffer Gradient Fractionator into 30–31 fractions of 15 drops each (0.13 mL) and the radioactivity in the first 20 fractions was determined. The total number of α-[^{125}I]BuTx binding sites present in the sample was obtained by summing the ^{125}I counts per minute in the single peak of radioactivity sedimenting at 9.5 S (around fractions 10–11). For routine assays the specific binding (i.e., that α-[^{125}I]BuTx binding that is protectable by 0.1 mM *d*-tubocurarine) was not determined. For more precise estimates of the AcChR activity in a sample, duplicates were performed and a sample pretreated with 0.1 mM dTC was run. In extracts of the BC_3H1 cells and in the purified BC_3H1 AcChR preparations, 90% of the α-[^{125}I]BuTx binding could be inhibited with 0.1 mM dTC. For the specific activity measurements, triplicate α-[^{125}I]BuTx binding assays were performed.

Purification of Acetylcholine Receptor. The AcChR from *Electrophorus electricus* was purified according to Lindstrom and Patrick (1974) and had a final specific activity of 3–4 nmol of toxin binding sites per mg of protein. The AcChR from *Torpedo californica* was purified in a similar fashion.

The purification of the AcChR from BC_3H1 cells was performed as follows and is described for a preparation starting with 5 g wet weight of BC_3H1 cells. All operations, unless otherwise noted, were performed at 0–4 °C, all buffers contain 0.1 mM phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$, Sigma Chemical Co.) and 50 units per mL of Trasylol (Aprotinin, Mobay Chemical Co.), and all glassware was treated with Siliclad (Clay Adams) prior to use. The cells were homogenized in 30 mL of PBS in a Dounce homogenizer and the extent of cell lysis was followed by visual examination of a few drops of the homogenate under a light microscope. The disrupted cells were sedimented at 105 000*g* for 1 h in a Type 40 rotor. After centrifugation the supernatant (Table I, PBS homogenate) was removed and the crude membrane pellet (containing membranes, nuclei, and mitochondria) was resuspended in 15 mL of PBS containing 3% (w/v) Triton X-100 (3% PBS homogenate or Triton extract) and incubated in the cold with gentle stirring for 90 min. The Triton extract (Table I, line 2) was then sedimented at 105 000*g* for 1 h in a Type 40 rotor. After centrifugation the lipid at the top of the centrifuge tube was removed by aspiration and the 3% Triton extract was passed over an 0.8 × 5 cm column packed with glass wool to remove the last traces of insoluble material. The Triton extract (16–18 mL) was mixed with 3.5 mL of packed agarose α-NTx in a 2.5 × 8.0 cm screw-capped vial and incubated in the cold with slow stirring for 3–4 h. At the end of the incubation the slurry was transferred to an 0.8 × 12 cm column and washed with 150–200 mL of 0.5% PBS. The column was next washed with 40–50 mL of 0.5% PBS containing 1 M NaCl and finally washed with 25 mL of 0.5% PBS. The batchwise adsorption of the AcChR in the 3% Triton X-100 homogenate to the agarose α-NTx affinity column resulted in the retention of 85 ± 11% (n = 11) of the toxin binding sites (Table I, line 3).

Elution of the bound BC_3H1 AcChR was accomplished with 0.5% PBS containing 1 M carbamoylcholine plus 1 mg per mL bovine serum albumin. Seven milliliters of the eluting buffer was added to the column and mixed evenly with the agarose and incubation was allowed to proceed at room temperature for 45 min. One column volume (3.5 mL) was removed and the incubation was continued for another 45 min. The second column volume of eluting buffer was removed and pooled with the first eluate. This carbamoylcholine eluate (Table I, line 4) was then dialyzed against 3–2 L changes of 0.5% PBS, concentrated in an Amicon ultrafiltration apparatus equipped with a PM 10 filter to a volume of approximately 1 mL, and loaded on 5–20% sucrose density gradients in PBS plus 0.2% (w/v) Brij-35. The gradients were centrifuged at 37 000 rpm for 21 h in an SW 41 rotor at 2 °C. A parallel SW 41 gradient tube contained a small portion of the dialyzed, concentrated carbamoylcholine eluate which had been incubated with α-[^{125}I]BuTx for 1 h.

The gradients were fractionated on a Gilson Micro Fractionator into 40 fractions (18 drops per fraction, 0.32 mL per fraction) and pooled on the basis of the α-[^{125}I]BuTx radioactivity profile in the parallel gradient. This SW 41 gradient pooled material (Table I, line 5) was dialyzed against 0.5% PBS, concentrated in an Amicon ultrafiltration device, and stored frozen in liquid nitrogen. Acetylcholine receptor stored in this manner will keep its α-BuTx binding activity for several months; however, repeated freezing and thawing result in a marked decrease in α-BuTx binding capacity.

Preparation of [^{3}H]Leucine-Labeled BC_3H1 Acetylcholine Receptor. BC_3H1 cells were plated on 60-mm tissue culture dishes and on day 7 after plating the medium was removed from 36 dishes and replaced with 2 mL of Dulbecco modified Eagle's medium minus leucine plus 20% dialyzed fetal calf serum. The leucine concentration was adjusted to 8×10^{-7} M with unlabeled leucine and 50 μCi per mL of [^{3}H]leucine (57 Ci/mmol, Amersham Searle). Labeling was performed for 20 h. The optimum concentration of leucine (8×10^{-7} M, above) was determined by experiments that showed this concentration of leucine to be the minimum allowable without a decrease in the capacity for AcChR synthesis. In this way we could label

the BC_3H1 cells with the highest possible specific activity [^{3}H]leucine. The purification of the ^{3}H-labeled AcChR was performed exactly as described above for the unlabeled AcChR.

Preparation of Antisera. Goat anti-rabbit IgG and anti-*Electrophorus electricus* AcChR antisera were prepared as described earlier (Patrick et al., 1973). Rabbit anti-*Torpedo californica* AcChR antiserum was prepared using purified *Torpedo* AcChR. A female New Zealand white rabbit was given 100 μg of *Torpedo* AcChR in Freunds complete adjuvant (3 vol of antigen: 7 vol of adjuvant). Injections of 100 μL were made intradermally at multiple sites along the right portion of the back. A booster injection of 100 μg of AcChR was given at day 14. Positive serum was obtained at day 16 and at the first sign of weakness or paralysis (Patrick and Lindstrom, 1973) the rabbit received a 0.2-mL intramuscular injection of a sterile solution of 370 μg per mL of neostigmine bromide and 15 μg per mL of atropine sulfate in 0.8% saline. The injections were repeated every 6–8 h for the next 4 days when the rabbit ceased to exhibit the paralysis which accompanies immunization with acetylcholine receptors. Forty milliliters of whole blood was removed on day 20 and again on day 26 and a final bleeding and sacrifice were performed on day 31. Anti-*Torpedo* AcChR antiserum was titered as described below.

Rabbit anti-BC_3H1 AcChR antiserum was prepared as follows. The SW 41 gradient fractions containing the AcChR from 3 preparations (~16 mL) were pooled, dialyzed to remove the sucrose, and concentrated to a final volume of 1.2 mL. A portion of this was removed and assayed for α-[^{125}I]BuTx binding activity just prior to injection. The pooled BC_3H1 AcChR was mixed with Freunds complete adjuvant (3 vol of antigen: 7 vol of adjuvant) and emulsified. The antigen–adjuvant mix (3.8 mL) was injected intradermally at 25–30 sites across the rabbit's back. The rabbit received 0.115 nmol of α-[^{125}I]BuTx binding sites of BC_3H1 AcChR (~30 μg total) in the single injection. The first immune serum was obtained on day 16 and the neostigmine–atropine injection schedule described for the anti-*Torpedo* AcChR antiserum was initiated. Forty milliliters of blood was removed on day 25 and again on day 30 and a final bleeding and sacrifice were performed on day 35.

Interaction of the BC_3H1 Acetylcholine Receptor with Plant Lectins. Stock solutions of the various lectins made in PBS minus Ca^{2+} and Mg^{2+} containing 0.5 M NaCl were prepared fresh for each experiment. The concentrations of the stock solutions were determined spectrophotometrically after filtration through a 0.22 μm pore diameter Millipore filter. All lectins were obtained from Miles Laboratories, Ltd. Succinylconcanavalin A was the generous gift of Dr. Robert Bloch. The interaction of various lectins and the BC_3H1 AcChR was assessed in 3 ways: (1) by chromatography of the BC_3H1 AcChR on agarose A-50m lectin conjugates, (2) by lectin inhibition of α-[^{125}I]BuTx binding to whole BC_3H1 cells, and (3) by lectin inhibition of α-[^{125}I]BuTx binding to the purified BC_3H1 AcChR. Details of the first method were described earlier (Patrick et al., 1977). To test the possible effects of lectins on the binding of α[^{125}I]BuTx to the AcChR in the BC_3H1 cells, the following protocol was used. Tissue culture dishes of differentiated BC_3H1 cells were removed from the incubator and washed twice with Prep medium and once with Prep medium plus 1 mg per mL BSA. Lectins were added to final concentrations of between 20 and 250 μg per mL and at various times the reaction was stopped by removal of the medium and washing with Prep medium containing 0.2% FCS. To each dish was then added α-[^{125}I]BuTx (final concentration 2×10^{-8} M) and the incubation was continued for 30 min. The cells were washed and harvested and the amount of bound radioactivity was determined. All assays were done in duplicate and were corrected for the nonspecific binding in control cultures containing 0.1 mM dTC. For studies on the effect of lectins on α-[^{125}I]BuTx binding to purified BC_3H1 AcChR, the following protocol was used. Aliquots of the BC_3H1 AcChR were incubated with the various lectins for 1 h at room temperature. The molar ratio of lectin to α-BuTx binding sites was approximately 500:1 in each case. Each sample then received α-[^{125}I]BuTx (final concentration 2×10^{-8} M) and the incubation was continued for 1 h at 4 °C. Finally, each sample received unlabeled α-BuTx (final concentration 2×10^{-6} M) and was adjusted to 50 mM with competing carbohydrate specific for each lectin. This last step served to remove any bound lectin from the BC_3H1 AcChR which might affect its sedimentation properties in the sucrose gradients. A portion of each sample was then applied to 5–20% sucrose density gradients and sedimented at 45 000 rpm in an SW 60 rotor for 15 h at 2 °C. Fractions were collected and the radioactivity determined as above. A control sample was also prepared which contained no added lectin. The specificity of the inhibition was determined by preparing samples to which a lectin-specific competing carbohydrate had been added prior to addition of the lectin.

Radioimmunoassays. Radioimmunoassays were performed in 1.5-mL Eppendorf tubes and the final volume of all assays was adjusted to 200 μL with 0.5% PBS. To each tube was added an aliquot of acetylcholine receptor and α-[^{125}I]BuTx (final concentration 2×10^{-8} M). After 60 min, dilutions of the appropriate antibody were added and the tubes returned to the cold for 8–12 h. Rabbit IgG and goat anti-rabbit IgG were then added and the incubation continued for 12–15 h. The precipitate that formed was pelleted by centrifugation for 2 min at 12 000*g* in a Brinkman Model 3200 centrifuge. The supernatant was removed by aspiration and the pellet washed twice with 1-mL aliquots of 0.5% PBS. After the final washing the pellet was dissolved in 200 μL of 0.1 N NaOH and a 100-μL portion was assayed for radioactivity. A control tube containing preimmune rabbit serum or, in the case of the BC_3H1 AcChR antiserum, rabbit serum from a littermate immunized with Freunds complete adjuvant but no BC_3H1 AcChR, was always subtracted from the experimental values to correct for the α-[^{125}I]BuTx nonspecifically bound to the immune precipitate.

Results

Purification of the Acetylcholine Receptor from the BC_3H1 Cell Line. A summary of the purification procedure and the yield of BC_3H1 AcChR at each stage of the purification is presented in Table I. The BC_3H1 cell line has proven to be an excellent source of mammalian acetylcholine receptor as the differentiated cells synthesize 42 ± 14 (mean ± standard deviation) (n = 8) pmol of α-[^{125}I]BuTx binding sites per g wet weight of cells. Also, the specific activity of the AcChR in 3% Triton X-100 extracts of the BC_3H1 cells is 2.3 ± 0.7 (n = 4) pmol of α-[^{125}I]BuTx binding sites per mg of cell protein. This specific activity is 2- to 3-fold higher than that reported for crude extracts of fetal calf skeletal muscle [approximately 1 pmol per mg of protein (Merlie et al., 1975)] and at least 15-fold higher than that reported for denervated rat diaphragm muscle homogenates [0.03 pmol of junctional AcChR per mg of protein, 0.17 pmol of extrajunctional AcChR per mg of protein (Brockes and Hall, 1975a)]. Even with these relatively high levels of AcChR in the BC_3H1 cells, the yields of purified AcChR [15 ± 7%, n = 7; see Table I, line 5] are low and un-

TABLE I: Purification Summary.[a]

Step	Vol (mL)	pmol of α-[125I]BuTx binding sites		mg of protein		Yield (%)	Sp[b] act.	Antibody[c] titer
		Per mL	Total	Per mL	Total			
1. PBS homogenate	35	0.4[d]	14	0.6	21			
2. 3% Triton X-100 homogenate	17.5	13.5	236	9.0	158	100	1.5	1.00
3. Affinity column flow through	17.5	2.0	35	7.4	133	86	N.D.	
4. Carbamoylcholine eluate	9.6	8.3	80	N.D.[e]		34	N.D.	0.95
	1.1	70.0	74			31		
5. SW 41 gradient pool	4.6	8.7	40	N.D.		17		
	0.93	32.3	30			13[f]	2600[g]	0.87

[a] The data presented in this table were obtained from a purification commencing with 6.4 g wet weight of BC_3H1 cells and are roughly proportional to results obtained from 11 preparations starting with varying amounts of the BC_3H1 cells. Lines 4 (carbamoylcholine eluate) and 5 (SW 41 gradient pool) show two values for each parameter measured: the data in the uppermost line represent values obtained prior to dialysis and/or concentration of the sample; the values in the lower line were obtained after dialysis and concentration of the sample. In all cases the yield was based on the number of picomoles of α-[125I]BuTx binding sites present in the 3% Triton X-100 homogenate. [b] The specific activity is in picomoles of α-[125I]BuTx binding sites per mg of total protein. [c] At each stage of the purification an aliquot of the AcChR was used as antigen in a radioimmunoassay using anti-BC_3H1 AcChR antiserum. The titer of the antiserum in the 3% Triton X-100 homogenate was set to 1.00. [d] Although no α-[125I]BuTx binding activity could be detected in the PBS homogenate, the values presented represent the minimum that could be detected. [e] The protein content of the carbamoylcholine eluate could not be determined as the eluting buffer contained 1 mg per mL of BSA. Analysis of the distribution of [3H]leucine radioactivity in the carbamoylcholine eluate of 3H-labeled AcChR (see Figure 1) demonstrates that 50% of the total radioactivity migrates at a position corresponding to 9.5×10^{-13} s. [f] Average yield was 15 ± 7% (mean ± standard deviation) ($n = 7$) with a high of 25% and a low of <5%. [g] The specific activity presented in this table was not measured with this preparation but was obtained from another purification and represents the highest value obtained (average of 2200, $n = 2$).

predictable (high of 25% and a low of <5% of the AcChR in the initial 3% Triton X-100 extract).

A contributing factor to the overall low yields has been the inability to elute all of the bound AcChR from the agarose-α-NTx affinity column (see Table I, lines 2 and 4). The amount of bound BC_3H1 AcChR that is eluted with 1 M carbamoylcholine is usually only 30–35%. Similar results have been reported for most AcChR purification procedures which employ a toxin affinity column as part of the purification scheme (Schmidt and Raftery, 1973; Klett et al., 1973; Lindstrom and Patrick, 1974; Merlie et al., 1975). Results using a different agonist (e.g., 1 mM benzoquinonium), longer elution times [e.g., 10–12 h (Merlie et al., 1975)], α-NTx coupled to polyacrylamide and to glass beads derivatized with long-chain alkylamines, or recycling the elution buffer through the affinity column (as suggested by Ong and Brady, 1974) have not significantly improved the yields of BC_3H1 AcChR at this step. We find that the most consistent results are obtained by using a low concentration of α-NTx coupled to agarose (0.1 mg of α-NTx per mL of packed agarose), restricting the time used to activate the agarose to 8–10 min (Cuatrecasas, 1970), and by including 1 mg of bovine serum albumin per mL in the elution buffer. In addition, we found that the best results were obtained by using a fresh batch of agarose-α-NTx for each purification, using siliconized glassware to minimize AcChR losses due to adsorption and to minimize all dialysis times.

At each stage of the purification a portion of the BC_3H1 AcChR was removed and incubated with α-[125I]BuTx and the complexes were used as antigen in a radioimmunoassay with anti-BC_3H1 AcChR antiserum (see Table I and footnote *c*). The titer of the antiserum with the receptor in the initial 3% Triton X-100 extract was arbitrarily set to 1.00. It is clear that as the purification proceeds the apparent titer of the antiserum decreases by some 13%. It is likely that the observed decrease in antibody titer reflects a decrease in the ratio of active to inactive BC_3H1 AcChR's as the purification proceeds. As the antibody cannot distinguish between antigenic determinants on active or inactive receptors, the presence of inactive receptors would result in a decrease in apparent antibody titer.

Assuming a final specific activity of 2600 pmol of α-[125I]BuTx binding sites per mg of BC_3H1 AcChR protein (see Table I, line 5, and Materials and Methods), it is possible to purify approximately 2–3 μg of BC_3H1 AcChR from 1 g wet weight of cells. As the purification procedure is rapid (approximately 55 h) and relatively simple, the limiting factor in isolating large amounts of purified BC_3H1 AcChR for biochemical analyses continues to be the availability of cells.

Estimation of Purity. The generally accepted criterion for purity of a protein is that it be homogeneous with respect to both size and charge. With the extremely small amounts of purified BC_3H1 AcChR available, however, it was impossible to directly demonstrate that the purified receptor satisfied either of these requirements. In an effort to estimate the purity of theBC_3H1 AcChR, we first determined its specific activity. Although a high specific activity would be consonant with a pure preparation it could not be taken as sufficient evidence for purity. The highest specific activity obtained for the BC_3H1 AcChR was 2600 pmol per mg of protein. This value is about 25–50% of that reported for the AcChR's from electric organs. For mammalian AcChR's, values of 6000 pmol per mg for denervated cat skeletal muscle AcChR (Dolly and Barnard, 1975) and 530 pmol per mg for rat extrajunctional AcChR's (Brockes and Hall, 1975a) have been reported.

A more rigorous demonstration of the purity of the BC_3H1 AcChR was performed using BC_3H1 cells grown in the presence of [3H]leucine. The BC_3H1 AcChR was purified from these cells as described in Materials and Methods for the unlabeled receptor preparations. Figure 1 is a sucrose density gradient profile of the [3H]leucine-labeled material eluted from the agarose-α-NTx column with carbamoylcholine. An aliquot of the dialyzed carbamoylcholine eluate was incubated in α-[125I]BuTx and run in a parallel gradient. Approximately 50% of the total 3H radioactivity eluted from the α-NTx affinity column co-migrates with the peak of α-[125I]BuTx binding activity. The fractions with the highest α-BuTx binding activity were pooled, concentrated, dialyzed, and re-run on sucrose density gradients. Figure 2 shows (1) that greater than 95% of the 3H-labeled protein in the purified BC_3H1 AcChR preparation migrates as a single symmetrical peak and (2) that

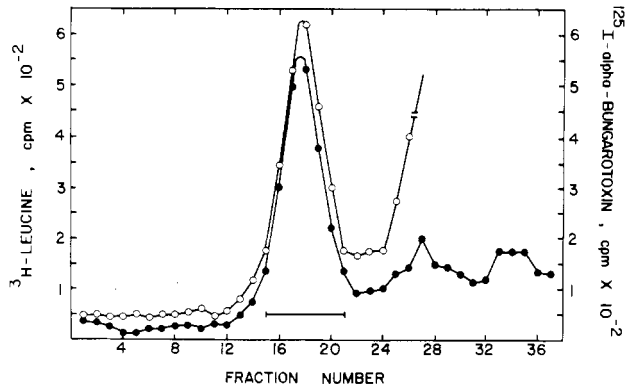

FIGURE 1: A sucrose density gradient profile of the [^{3}H]leucine-labeled BC_3H1 AcChR eluted with carbamoylcholine (●) from the agarose A-50m-α-neurotoxin affinity column and the α-[^{125}I]BuTx binding activity (○) of the same sample run in a parallel gradient. The labeling of the BC_3H1 cells with [^{3}H]leucine, the preparation of the Triton X-100 extract, and subsequent affinity chromatography are described in Materials and Methods. The carbamoylcholine eluate was dialyzed, concentrated, and applied to 5–20% sucrose density gradients in PBS containing 0.5% (w/v) Triton X-100. Centrifugation was performed in a SW 41 rotor at 37 000 rpm for 23 h at 2 °C. Fractions of 0.33 mL were collected from the bottom of each tube and 100-μL aliquots were counted for [^{3}H]leucine radioactivity. The parallel gradient containing the α-[^{125}I]BuTx labeled BC_3H1 AcChR was fractionated and counted in the same manner. Approximately 50% of the total [^{3}H]leucine radioactivity migrates at a position corresponding to 9.5 S. The bar indicates the fractions that were pooled. The specific activity of the α-[^{125}I]BuTx was 7.5 × 10^7 cpm per nmol.

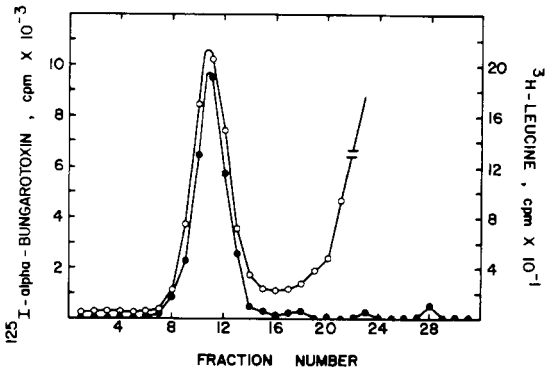

FIGURE 2: A sucrose density gradient profile of the SW 41 gradient purified [^{3}H]leucine-labeled BC_3H1 AcChR (●) and the α-[^{125}I]BuTx binding activity (○) of a portion of the purified ^{3}H-labeled BC_3H1 AcChR. The pooled ^{3}H-labeled BC_3H1 AcChR from the SW 41 gradients (see Figure 1) was dialyzed and concentrated in an Amicon ultrafiltration apparatus and a 200-μL aliquot was loaded on a 5–20% sucrose gradient in PBS containing 0.5% (w/v) Triton X-100. A parallel gradient contained 100 μL of the same sample incubated with α-[^{125}I]BuTx (final concentration 2 × 10^{-8} M) for 1 h prior to loading the sample on the gradient. The samples were sedimented at 50 000 rpm in an SW 60 rotor for 10 h at 2 °C. Fractions of 0.13 mL were collected as described in Materials and Methods and assayed for either [^{3}H]leucine or α-[^{125}I]BuTx radioactivity. Greater than 95% of the [^{3}H]leucine radioactivity is found at a position corresponding to 9.5 S. The specific activity of the α-[^{125}I]BuTx was 7.4 × 10^7 cpm per nmol.

all of the α-[^{125}I]BuTx binding activity co-migrates with the purified AcChR. Within the limits of this technique, then, the BC_3H1 AcChR appears to be homogeneous with respect to size. Because of the small amount of ^{3}H-labeled BC_3H1 AcChR available (as well as several technical problems), it has been impossible to analyze the native molecule for charge homogeneity by polyacrylamide gel electrophoresis.

Determination of the Hydrodynamic Properties of the BC_3H1 Acetylcholine Receptor. Sucrose density gradient analyses of α-[^{125}I]BuTx labeled BC_3H1 AcChR (data not

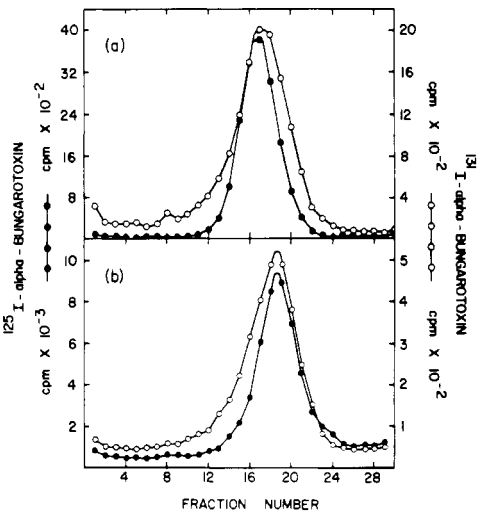

FIGURE 3: A comparison of the purified BC_3H1 acetylcholine receptor with "surface" (a) and "hidden" (b) acetylcholine receptors from the BC_3H1 cell line. In both a and b the purified BC_3H1 AcChR was labeled with α-[^{131}I]BuTx (○) and the "surface" AcChR (a) or "hidden" AcChR (b) with α-[^{125}I]BuTx (●). The "surface" AcChR was prepared as follows: 60-mm culture dishes of differentiated BC_3H1 cells were removed from the incubator, washed twice with Prep medium containing 0.1% fetal calf serum, and incubated with 2 × 10^{-8} M α-[^{125}I]BuTx for 30 min at room temperature. The α-[^{125}I]BuTx was then removed and the cells washed three times with PBS, harvested, and dissolved in 3% (w/v) Triton X-100 in PBS. Excess unlabeled α-BuTx was added to 2 × 10^{-6} M and the extract was incubated at 4 °C for 1 h. The "hidden" BC_3H1 AcChR was labeled as follows: 60-mm culture dishes of differentiated BC_3H1 cells were removed from the incubator, washed twice with Prep medium plus 0.1% fetal calf serum, and incubated with 2 × 10^{-8} M unlabeled α-BuTx for 30 min. The α-BuTx was removed from the dishes and cells were washed 3 times with PBS, harvested, and dissolved in 3% (w/v) Triton X-100 in PBS. The Tritor X-100 extract was then incubated with 2 × 10^{-8} M α-[^{125}I]BuTx for 1 h at 4 °C. Unlabeled α-BuTx was then added to 2 × 10^{-6} M and the incubation continued for an additional hour. A portion of the α-[^{125}I]-BuTx-labeled "surface" and "hidden" AcChR extracts was mixed with the α-[^{131}I]BuTx-labeled purified BC_3H1 AcChR and applied to 5–20% sucrose density gradients and sedimented at 50 000 rpm for 10 h in an SW 60 rotor at 2 °C. Thirty fractions of 10 drops each (0.09 mL) were collected and the ^{125}I and ^{131}I radioactivities in each fraction were determined.

shown) yield an apparent sedimentation coefficient for the BC_3H1 AcChR of 9.5 × 10^{-13} s. This value is quite similar to that reported for purified rat diaphragm muscle AcChR [9 S (Brockes and Hall, 1975b)] and for the AcChR purified from the electric organ of the eel [9.5 S (Lindstrom and Patrick, 1975) and 9 S (Meunier et al., 1974)].

Two classes of acetylcholine receptor are present in embryonic chick thigh muscle (Devreotes and Fambrough, 1975) and BC_3H1 cells (Patrick et al., 1977): a "surface" AcChR that is readily labeled with α-[^{125}I]BuTx added to the external medium and a "hidden" AcChR that binds α-BuTx only after solubilization of the cells with detergent. While the "hidden" and "surface" AcChR's from BC_3H1 cells are indistinguishable with anti-eel AcChR antiserum and both contain carbohydrate, they do differ with respect to their apparent sedimentation coefficients in sucrose density gradients (Patrick et al., 1977). The "surface" AcChR from the BC_3H1 cells has an s value 0.5–0.6 × 10^{-13} s larger than the "hidden" AcChR. As the BC_3H1 AcChR was purified from detergent extracts of whole cells, we wanted to determine if both "surface" and "hidden" AcChR's were present in the purified preparations or if only one of the two classes of AcChR was present. Figure 3 shows the results of a sucrose density gradient analysis of α-[^{131}I]BuTx-labeled purified BC_3H1 AcChR which had been mixed with (a) α-[^{125}I]BuTx-labeled "surface" or (b) α-[^{125}I]BuTx-labeled "hidden" receptor just prior to application

to the gradients. If the purified BC_3H1 AcChR represents a mixture of both "surface" and "hidden" AcChR's then one would predict that in the case of a mixture of ^{125}I-labeled "surface" and ^{131}I-labeled purified AcChR, the leading edge of the ^{125}I and ^{131}I peaks of radioactivity should coincide but the ^{131}I peak of radioactivity should have a trailing edge not coincident with the trailing edge of the ^{125}I-labeled "surface" receptor. In the case of a mixture of ^{125}I-labeled "hidden" and ^{131}I-labeled purified AcChR, the leading edge of the ^{131}I peak of radioactivity should be displaced toward the bottom of the centrifuge tube (relative to the leading edge of the ^{125}I peak) while the trailing edge of both peaks of radioactivity should be coincident. An analysis of the distribution of the ^{131}I and ^{125}I radioactivity profiles in Figure 3 suggests that the purified BC_3H1 AcChR preparations contain a mixture of both "surface" and "hidden" AcChR's and that the differences in apparent sedimentation coefficients of the "surface" and "hidden" AcChR's observed in crude extracts of BC_3H1 cells are maintained throughout the purification procedure.

The Subunit Composition of Purified BC_3H1 Acetylcholine Receptor. Figure 4 is a densitometer scan of Coomassie brilliant blue stained BC_3H1 AcChR after electrophoresis in 7.5% polyacrylamide gels containing $NaDodSO_4$. The sample of purified BC_3H1 AcChR was prepared and the electrophoresis performed as described in Materials and Methods. The BC_3H1 AcChR appears to be an oligomer with subunit molecular weights of 72 000, 65 000, 53 000, and 44 000.

The Interaction of the BC_3H-Acetylcholine Receptor with Plant Lectins. The AcChR's purified from both *Electrophorus electricus* (Meunier et al., 1974) and denervated rat diaphragm muscle (Brockes and Hall, 1975b) have been shown to bind the plant lectin concanavalin A. This lectin, which binds specifically and reversibly to oligosaccharides and glycoproteins containing α-D-glucopyranosyl and/or α-D-mannopyranosyl moieties, has been a valuable tool in establishing that the AcChR's from these sources are glycoproteins. Furthermore, the *Electrophorus* AcChR binds lectins from *Phaseolus vulgaris* which suggests that it also contains covalently bound *N*-acetyl-D-galactosamine (Meunier et al., 1974). Patrick et al. (1977) have shown that the AcChR in 3% Triton X-100 extracts of BC_3H1 cells was retained on Con A–agarose conjugates but was not retained on wheat germ agglutinin–agarose conjugates. These data suggest that the BC_3H1 AcChR is also a glycoprotein with glucosyl and/or mannosyl residues but no detectable *N*-acetyl-D-glucosamine residues. We have found that the purified BC_3H1 AcChR is quantitatively retained on Con A–agarose conjugates but is not retained on agarose conjugates of fucose binding protein (D-fucose), soybean agglutinin (D-galactose, *N*-acetyl-D-galactosamine), or wheat germ agglutinin (*N*-acetyl-D-glucosamine). The purified BC_3H1 AcChR is similar with respect to carbohydrate content to the AcChR's from denervated rat diaphragm muscle and the electric eel but, unlike the AcChR from eel, it contains no detectable *N*-acetyl-D-galactosamine residue. It should be noted that the interaction of the BC_3H1 AcChR with Con A is sufficient evidence for the presence of glucosyl and/or mannosyl residues on the receptor but that a negative result (by this test) with the other lectins cannot be taken as conclusive evidence for the absence of a given carbohydrate.

Meunier and co-workers (1974) noted that the binding of α_1-isotoxin (*Naja nigricollis*) to purified eel AcChR was inhibited by Con A. We have tested the ability of various plant lectins to inhibit the binding of α-[^{125}I]BuTx to the AcChR in intact BC_3H1 cells as well as to the purified BC_3H1 AcChR. We have found that incubation of the BC_3H1 cells for 60 min in the presence of 25 μg per mL of Con A results in an average

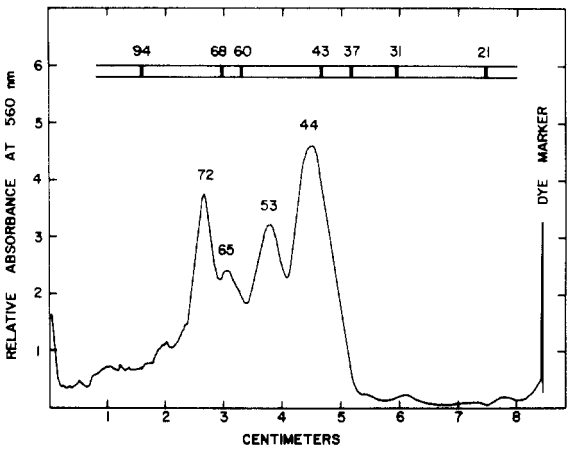

FIGURE 4: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of purified BC_3H1 acetylcholine receptor. The sample was prepared and electrophoresis performed as described in Materials and Methods. The positions of the molecular weight standards in a parallel gel are represented in the insert above the densitometer tracing. The numbers are apparent molecular weights × 10^{-3}. Molecular weight standards employed were: phosphorylase *a* (94 000), bovine serum albumin (68 000), L-arabinose isomerase (60 000), ovalbumin (43 000), glyceraldehyde-phosphate dehydrogenase (37 000), carbonic anhydrase (31 000), and myokinase (21 000).

reduction of 35% (n = 8) in the toxin binding capacity of the cells. This level of inhibition is not increased at higher concentrations of Con A (up to 250 μg per mL) or by incubating the cells in Con A for longer periods of time (up to 2.5 h). The Con A inhibition of toxin binding is completely blocked by 50 mM α-methyl-D-mannoside and the inhibition is reversible if, after incubation in Con A, the cells are treated with α-methyl-D-mannoside for 30 min prior to addition of α-[^{125}I]BuTx. The purified BC_3H1 AcChR is also sensitive to Con A inhibition of α-BuTx binding. In fact, greater than 95% of the α-[^{125}I]BuTx binding activity of purified BC_3H1 AcChR is lost after incubation with Con A. Control experiments with α-methyl-D-mannoside as a competitive inhibitor of Con A show that this inhibition is also specific and reversible. Fucose binding protein, wheat germ agglutinin, soybean agglutinin, and succinyl-Con A do not inhibit α-BuTx binding to either whole cells or purified BC_3H1 AcChR. However, the addition of succinyl-Con A to preformed α-[^{125}I]BuTx–AcChR complexes results in an increase in the sedimentation coefficient of the toxin–receptor complexes (data not shown). This suggests that while succinyl-Con A does not interfere with toxin binding, the lectin does bind to the BC_3H1 AcChR.

Preparation of a Rabbit Anti-BC_3H1 Acetylcholine Receptor Antiserum. Although antisera prepared against AcChR's purified from *Electrophorus electricus* and *Torpedo californica* are available, the cross-reactivity of these sera against the BC_3H1 AcChR is low (see Table II). Since an antiserum directed against the BC_3H1 AcChR would be a valuable probe of receptor structure and function in the BC_3H1 cell line, we prepared such an antiserum using the purified BC_3H1 acetylcholine receptor.

With the extremely small amounts of BC_3H1 AcChR available (5–8 μg of AcChR protein per preparation) it was necessary to pool several preparations to generate enough receptor to initiate a realistic immunization schedule. Our first efforts involved absorbing the purified receptor to DE-52 anion exchange resin and mixing the resin with Freund's complete adjuvant prior to injection. As highly polymerized or particulate antigens are often extremely immunogenic, it was thought that absorption to the small resin particles would in-

crease the likelihood of an immune response to the low levels of injected BC_3H1 AcChR. Two attempts with this approach failed to yield a positive antiserum. A second approach was used in which a mixture of the receptor in Freund's complete adjuvant was injected intradermally at 25–30 sites across the back of the rabbit (see Materials and Methods for complete details). This approach resulted in a positive antiserum with recent lots of antiserum having titers as high as 0.29 nmol of α-[^{125}I]BuTx binding sites per mL of antiserum. Table II shows the results of a radioimmunoassay performed to determine if antisera prepared against AcChR's purified from *Electrophorus electricus* and *Torpedo californica* cross-react with the BC_3H1 AcChR. Although the cross-reactivity of these sera with the BC_3H1 AcChR was low, the data show that acetylcholine receptors from all three sources share some antigenic determinants.

TABLE II: The Immunological Cross-Reactivity of Various Anti-Acetylcholine Receptor Antisera with the BC_3H1 Acetylcholine Receptor.[a]

Source of AcChR	Antiserum prepared against: Eel AcChR	Antiserum prepared against: Torpedo AcChR
BC_3H1 cell line	4% (2.3)	7% (0.90)

[a] The percent immunological cross-reactivity was obtained by: (antiserum titer on BC_3H1 AcChR)/(antiserum titer on homologous AcChR)] × 100. The numbers in parentheses are the titers of the antisera on homologous AcChR in nanomoles of α-[^{125}I]BuTx binding sites precipitable per mL of antiserum.

Discussion

In this paper we have presented the purification and partial characterization of a nicotinic acetylcholine receptor from the mouse, nonfusing muscle cell line BC_3H1. Affinity chromatography of Triton X-100 solubilized membranes on an agarose–α-NTx affinity column followed by sucrose density gradient centrifugation resulted in a 1700-fold purification of the α-bungarotoxin binding component. Although an accurate evaluation of the purity of the BC_3H1 AcChR is difficult, results using isotopically labeled receptor show that 95% of the radioactivity in purified [^{3}H]AcChR preparations migrates as a single symmetrical peak which co-sediments with the α-BuTx binding activity. Pharmacological (Patrick et al., 1977) and electrophysiological (Schubert et al., 1973) evidence suggests that the BC_3H1 AcChR is quite similar to that found in electric organs, vertebrate skeletal muscle, and in other muscle cell lines (Patrick et al., 1972). The BC_3H1 AcChR also appears to be structurally similar to nicotinic AcChR's from other sources. Reports to date demonstrate that nicotinic AcChR's are glycoproteins (Meunier et al., 1974; Brockes and Hall, 1975a; Raftery et al., 1976), have apparent sedimentation coefficients of 9–10 S (Meunier et al., 1974; Lindstrom and Patrick, 1974; Brockes and Hall, 1975b), and consist of several nonidentical subunits (Karlin and Cowburn, 1973; Schmidt and Raftery, 1973; Chang, 1974; Lindstrom and Patrick, 1974; Weill et al., 1974). Merlie et al. (personal communication) have purified an AcChR from fetal calf skeletal muscle grown in culture and have demonstrated by two-dimensional gel electrophoresis that their receptor preparations contain a single major polypeptide of approximately 41 000 daltons. Although the multiple subunit nature of the BC_3H1 AcChR is reminiscent of receptors purified from electric organ, it appears to have several high molecular weight components not present in fetal calf skeletal muscle AcChR. We have also presented evidence that AcChR's from both *Electrophorus electricus* and *Torpedo californica* share antigenic determinants with the BC_3H1 AcChR.

The studies presented here on the interaction of lectins with the AcChR in whole BC_3H1 cells and purified BC_3H1 AcChR serve to establish that the BC_3H1 AcChR is a glycoprotein which binds Con A. It is unlikely that the receptor contains only glucosyl and/or mannosyl residues but until sufficiently sensitive techniques are available, the exact nature of the carbohydrate content will remain uncertain. The Con A inhibition of α-BuTx binding to the AcChR in whole BC_3H1 cells suggests that some of the carbohydrate on the AcChR is exposed to the external milieu. In the simplest terms, Con A might bind to the carbohydrate on the receptor and physically occlude the α-BuTx from its binding site. It is not clear why Con A binding to the AcChR in whole BC_3H1 cells does not result in complete inhibition of α-[^{125}I]BuTx binding as it does with the purified BC_3H1 AcChR. Even with an excess of Con A (100-fold excess of Con A to total Con A binding sites on the BC_3H1 cells) the maximum inhibition of α-BuTx binding never exceeds 35%. It is possible that the carbohydrate on the solubilized BC_3H1 AcChR is in a configuration more susceptible to interaction with Con A and subsequent inhibition of α-BuTx binding. In the cell membrane, however, the carbohydrate moiety on the AcChR may exist in different states, one of which will interact with Con A and inhibit toxin binding and another which is refractory to Con A inhibition of toxin binding. We have no evidence that the carbohydrate moiety on the BC_3H1 AcChR is involved in receptor function; indeed, Con A does not block the response of BC_3H1 cells to iontophoretically applied acetylcholine (B. Brandt, personal communication) nor does treatment of the cells with periodate affect α-BuTx binding (unpublished observation).

The observation that the purified BC_3H1 AcChR is a mixture of both "surface" and "hidden" receptors suggests that the factor(s) involved in the difference in sedimentation coefficients for the two classes of AcChR is stable and conserved throughout the purification procedure. As the difference in s values for the "surface" and "hidden" AcChR's (Patrick et al., 1977) was found with cells in which the "surface" receptors had been labeled with α-BuTx prior to extraction with Triton X-100, it could be argued that the binding of the toxin stabilized the "surface" receptors during preparation of the extracts (Devreotes et al., 1977). We have shown this possibility to be unlikely as the purified BC_3H1 AcChR preparation contains both "surface" and "hidden" receptors and was obtained from cells solubilized with detergent in the absence of α-BuTx.

Finally, a rabbit anti-BC_3H1 AcChR antiserum has been prepared which should prove useful for investigations of the AcChR in the BC_3H1 cell line. Labeling of this antiserum with fluorescent or electron-dense markers should make possible experiments designed to explore the distribution, orientation, and topology of both the subcellular and plasma membrane bound AcChR's. In addition, such antisera may be useful for obtaining mutants of the BC_3H1 cell line which are missing some or all of the antigenic determinants found in the native receptor. Such mutants could be instrumental in establishing direct correlations between structure and function of the BC_3H1 acetylcholine receptor.

Acknowledgments

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Effects of Neuraminidase on Lectin Binding by Wild-Type and Ricin-Resistant Strains of Hamster Fibroblasts†

Saul W. Rosen*,‡ and R. Colin Hughes

ABSTRACT: The nature of cell surface receptors for ricin on wild-type and ricin-resistant variants of baby hamster kidney fibroblasts has been studied. Neuraminidase stimulated ricin binding threefold by wild-type cells, and increased their susceptibility to ricin toxicity as measured by inhibition of [^{3}H]leucine uptake (LD_{30} fell from 5.0 to 0.5 μg/mL). Basal ricin binding by ricin-resistant variants (10–300% that of wild type) was also stimulated (2- to 17-fold) by neuraminidase in all seven clonal strains examined; susceptibility to ricin was greatly increased by neuraminidase in these variants. Neuraminidase did not affect the binding of concanavalin A by wild type or a ricin-resistant variant, but decreased the binding of wheat-germ agglutinin by 90% in both cell types. The trivial binding of peanut agglutinin by wild type and a ricin-resistant variant was markedly enhanced (14- to 22-fold) by neuraminidase. Neither collagenase (50 U/mL) nor Pronase (0.0001%) affected ricin binding by wild type or a ricin-resistant variant. These data suggest the existence of "exposed" and "cryptic" oligosaccharide receptors for ricin on the cell membrane glycoproteins of baby hamster kidney fibroblasts. The cryptic ricin receptors probably include at least the sequence D-galactosyl-β-(1→3)-*N*-acetylhexosamine substituted by sialic acid residues. Exposed and cryptic ricin receptors appear to be different and under separate genetic control.

The isolation from baby hamster kidney cells of stable variants resistant to ricin, the toxin lectin of castor beans (*Ricinus communis*), has been reported from this laboratory (Meager et al., 1975, 1976). Evidence has been presented for two major phenotypic classes: variants binding normal amounts of ricin and variants that bind this lectin poorly. Binding of the lectin to cell surface receptors is necessary for the toxic effects on intact cells (Olsnes et al., 1974a,b; Nicolson et al., 1975b; Olsnes and Pihl, 1976) and a deficiency in surface receptors could alone account for resistance. Although the steps subsequent to initial binding are not fully understood, it is clear that the toxin must enter the cell, perhaps by an endocytosis-like

† From the Division of Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom. *Received April 28, 1977.*

‡ Present address: Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Md. 20014.