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Purification and Characterization of an Acetylcholine Receptor from Mammalian Skeletal Muscle†

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ABSTRACT: The acetylcholine receptor from denervated mammalian skeletal muscles has been purified 2400-fold. Receptor activity was followed by an assay based upon the binding of a [^{3}H]triacetyl derivative of α-bungarotoxin. Efficient extraction from the muscle of the native receptor was obtained in 1.5% Triton X-100, 15 h at 4 °C, in the presence of inhibitors of proteolysis. Gel filtration of the extract separated much other protein, including acetylcholinesterase, from the receptor. The next stage employed an affinity column containing an immobilized cholinergic (quaternary ammonium) ligand, with biospecific elution by 2 mM gallamine triethiodide. The final stages involved ion-exchange chromatography with a salt gradient elution and salt removal by continuous-flow dialysis, in 0.1% Triton X-100. The muscle receptor is a protein with 6000 nmol of toxin-binding sites per g of protein. It is homogeneous in gel electrophoresis. The receptor–toxin complex has an isoelectric point of pH 5.3. The association rate constant for the reaction with toxin, 2.9×10^5 M^{-1} s^{-1} at 25 °C (pH 8), is consistent with the rates for vertebrate muscle receptors in less pure forms reported elsewhere with related toxins. Protection from the toxin binding, exerted specifically by cholinergic ligands, leads to values of affinities for those ligands in agreement with their known pharmacological degrees of effectiveness.

Recent advances in the purification of the acetylcholine receptor (ACh.R)[1] have mainly been possible due to the discovery of snake polypeptide α-neurotoxins which bind specifically and virtually irreversibly to the nicotinic cholinergic receptor of fish electric organs and of vertebrate skeletal muscle (Chang and Lee, 1963; Changeux et al., 1970; Barnard et al., 1971; Miledi and Potter, 1971). A number of investigators have obtained preparations of ACh.R from electric organs, and shown it to be a glycoprotein that binds cholinergic ligands and inorganic cations. (For reviews, see Karlin et al., 1975; Changeux, 1975). In contrast, much less information is available on the biochemistry of the ACh.R from skeletal muscle, although its electrophysiology and pharmacology have been studied in great detail [reviewed, e.g., by Hubbard (1974) and Rang (1974)]. Therefore, it is important to isolate and characterize the ACh.R from mammalian muscle so that its biochemical properties can be correlated with, and used to fully interpret, the physiological data. Furthermore, it would enable

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[1] Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ACh.R, acetylcholine receptor; BuTX, α-bungarotoxin; [^{3}H]BuTX, [^{3}H]triacetyl-α-bungarotoxin; *d*-TC, *d*-tubocurarine; Flaxedil, gallamine triethiodide; $PhCH_2SO_2F$, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

a detailed study to be made of the ACh.R in muscles in various states, e.g., pre- and postinnervation, after denervation, in cell culture, or in certain muscle diseases. With regard to the latter, it is essential to have purified muscle ACh.R available for studies on antireceptor antibodies which appear to be involved in the etiology of a muscle weakness disease, myasthenia gravis (Patrick and Lindstrom, 1973). It is also desirable to establish how similar the molecular properties of ACh.R from muscle are to that from electric organs, so as to utilize the information on one source in relation to data available on the other.

Because of the relatively low amounts of the receptor in muscle, progress in its isolation has been slower than in the case of electroplaque. It has been shown that the reaction of this receptor with radioactively labeled BuTX can be specific enough to be used to determine the numbers of ACh.R sites per muscle endplate (Barnard et al., 1971; Miledi and Potter, 1971; Berg et al., 1972; Fambrough and Hartzell, 1972; Porter et al., 1973; Chang et al., 1973), and their densities at the ultrastructural level, per μm^2 of synaptic membrane (see Barnard et al., 1975). In fact, over 90% of the BuTX-binding sites in intact muscle, or in sarcolemmal membrane preparations and detergent extracts of the latter, are ACh.R sites, in that they have a high affinity for d-TC or ACh (Dolly and Barnard, 1974; Barnard et al., 1975, 1977). Two components that bind BuTX were solubilized from rat or mouse diaphragm muscles and shown to have apparent molecular weights (by gel filtration in 0.2% Triton X-100) of about 500 000 and 200 000, respectively (Chiu et al., 1973). The former is present in the endplate region only (in innervated fast-twitch muscles), and is thought to be the native ACh.R. A form of similar molecular weight is greatly increased in chronically denervated muscle (Miledi and Potter, 1971; Chiu et al., 1973). Supersensitivity to ACh is found all along the muscle membrane in that state, as well as in fetal muscle and myotubes in culture, and is attributable to toxin-binding ACh receptors. (For reviews, see Barnard et al., 1975; Changeux and Danchin, 1976).

This form of the ACh.R has recently been partially purified from cat denervated leg muscles, and shown to bind cholinergic agonists and antagonists with high affinities (Dolly and Barnard, 1974; Barnard et al., 1975). It has also recently been partly purified from denervated and innervated rat muscle (Brockes and Hall, 1975; Almon and Appel, 1976a). Possible differences between the ACh.R of denervated and normal muscle will be considered in the Discussion section of this paper. We can note here that the proliferation of an ACh.R in denervated muscle renders it easier to purify completely on a scale sufficient to characterize it as a protein. In view of its close similarity, when in situ, to the synaptic ACh.R, we believe that this muscle receptor offers a good guide to the molecular constitution of the nicotinic ACh.R. In this paper, therefore, we report the characterization and purification of the ACh.R from denervated rat diaphragm and leg muscles of the cat, in the latter case to homogeneity, using gel filtration, biospecific chromatography and ion-exchange chromatography. A preliminary report of some of these findings has been made (Dolly and Barnard, 1975).

Materials and Methods

Lyophilized venoms of *Bungarus multicinctus* and *Naja naja siamensis* were obtained from Miami Serpentarium, Miami. Sources of other materials were: [^{3}H]acetic anhydride, Amersham/Searle; Sepharose 6B and 4B, Pharmacia; Ultrogel AcA 22, LKB; Pellicon membrane (PSJM), Millipore Corp.; human serum albumin (electrophoretically pure), Behringwerke A.G., Marburg; Triton X-100 and Soluene-100, Packard; Flaxedil, May and Baker; all other chemicals, Sigma; hollow-fiber Minibeaker dialyzers, Bio-Rad Labs; Emulphogene BC-720 was a gift from General Aniline and Film Corp., New Jersey.

Labeling of BuTX. BuTX was purified to homogeneity from the venom of *Bungarus multicinctus* and acetylated with [^{3}H]acetic anhydride as described previously (Barnard et al., 1971). The amount of reagent was limited, such that substitution proceeded only to the mono-, di- and triacetylated stages. ^{3}H-labeled triacetylated BuTX was separated from the other labeled species by chromatography on a column (75 × 1.3 cm) of CM-cellulose (CM-52) equilibrated with 0.05 M ammonium acetate. The latter was generated by a 400-mL constant-volume mixing chamber and a reservoir which initially contained 0.05 and 0.4 M ammonium acetate, respectively. Protein content in the fractions was measured by the Lowry method using a solution of BuTX as a standard. Two adjacent ^{3}H-labeled triacetylated peaks (A and B) were separated, these being the earliest peaks to emerge in the chromatogram. Both isomeric forms, A and B (A being the more basic peak), gave the same results in reactions with the muscle receptors. The specific radioactivity of the labeled toxin was 4500 Ci/mol, in each form.

Labeling and Extraction of ACh.R. Rat or mouse diaphragm, rat soleus, and cat hind limb (lower) muscles were used; denervation was performed under anesthesia by scission of the phrenic or sciatic nerves (the three major branches of the latter, in the cat). The rats were killed by decapitation and the cats by intravenous pentabarbitone, 3 and 4 weeks, respectively, after nerve section. The dissected muscle was rinsed in, and then chopped finely in, 50 mM phosphate buffer (pH 8.0)/10^{-3} M EDTA at 4 °C, and centrifuged at 100 000*g*, 30 min. It was then extracted in the same medium containing 1.5% Triton X-100 or Emulphogene BC-720 (1 g of muscle wet weight per 2.5 mL of medium) on a shaking water bath at 4 °C and was centrifuged as above, the supernatant being used immediately. Except where noted, the detergent extraction was for about 15 h, with the medium previously deoxygenated by bubbling with O_2-free N_2 and with protease inhibitors present (see Results). The [^{3}H]BuTX–ACh.R complex was prepared by incubation of the detergent extract of ACh.R with [^{3}H]-BuTX (0.2 μg/mL) for 12 h at 4 °C. Labeling of the muscle receptor sites in situ was performed by shaking the intact muscles with [^{3}H]BuTX (5 μg/mL of Tyrode solution) for 3 h at 25 °C, followed by extensive washing with Tyrode solution. The muscles were then chopped and shaken at 25 °C for 30 min with unlabelled BuTX (30 μg/mL of Tyrode) and d-TC (10^{-4} M), to remove the loosely bound [^{3}H]BuTX and to prevent any further binding of labeled toxin, followed by repeated washings with Tyrode solution (Barnard et al., 1977). The [^{3}H]BuTX–ACh.R complex was then extracted as described above. All subsequent procedures were at 4 °C.

Assay of ACh.R Activity. A method based upon the binding of the ACh.R–[^{3}H]BuTX complex to a DEAE-cellulose filter disc, adapted from that used for *Electrophorus* receptor by Klett et al. (1973), was employed. A related method has been used by Schmidt and Raftery (1973) for *Torpedo* receptor. Activity was measured routinely at 37 °C using 20–60 nM [^{3}H]BuTX in 0.2% Triton X-100/10 mM phosphate buffer, pH 8.0, in a total volume of 120 μL. Heat-inactivated ACh.R (held at 100 °C for 2 min), or buffer, replaced the ACh.R in blank tubes. After incubation for 1 h, the reaction mixture (75–100 μL) was pipetted evenly onto a DEAE-cellulose (DE-81) paper disc resting on mounted pins. After standing for 2 min, the discs were washed (4 × 5 min) in a stainless steel mesh holder immersed in 200 mL of a stirred solution of 0.2%

Triton X-100/25 mM phosphate, pH 8.0. The discs were subsequently dried under an infrared lamp and counted in a toluene-based scintillation medium (Dolly et al., 1977a) containing 10% (v/v) Soluene-100 at 40% efficiency on a Packard 2425 spectrometer. Aqueous samples were counted similarly in a Triton/xylene-based medium (Anderson and McClure, 1973). [^{3}H]BuTX stock solutions (1–3 μg/mL) used for the assays were freshly thawed after being stored frozen in 0.2% Triton X-100/10 mM phosphate buffer, pH 8.0, in polypropylene (1.5 mL) tubes; the detergent prevented artefacts due to adsorption of the toxin onto the walls of the container.

Affinity Chromatography. Trimethyl(*p*-aminophenyl)-ammonium chloride was covalently attached to Sepharose 4B, through two lengths of the arm $-NH(CH_2)_3NH-(CH_2)_3NHCO(CH_2)_2CO-$, using the method of Berman and Young (1971). The equilibrating buffer for this resin was 0.2% Triton X-100/50 mM phosphate buffer (pH 8.0)/10^{-4} M EDTA. The gel-filtered receptor (see Results) was concentrated at least 10-fold, by ultrafiltration through a Millipore PSJM membrane under N_2. The concentrate was adjusted to pH 7.8–8.0 with solid K_2HPO_4 and loaded on the column; on the usual preparative scale, a 15-mL column was used, and the first wash was with 200 mL of 50 mM NaCl in the equilibrating buffer (or until no protein was detectable in the effluent), followed by 25 mM phosphate (pH 8)/0.2% Triton X-100/10^{-4} M EDTA (50 mL). Then in the latter medium, 2.1 mM Flaxedil (30 mL) was applied for displacement. This effluent was dialyzed by flowing through two hollow-fiber Minibeakers in series, against 0.2% Triton X-100 buffer solution, the latter pumped at 80 mL/min. (The resin was regenerated using 1 M NaCl after every run, and could then be reused a number of times.) The pooled peak fractions were applied to a column (1 mL) of DEAE-Sephadex in 25 mM phosphate (pH 8)/0.1% Triton X-100, and washed with 0.1 M NaCl in that medium. When protein elution ceased, the receptor was eluted with a linear gradient of 0.1–1.2 M NaCl (20 mL), using dialysis as noted above but against 0.1% Triton X-100.

Gel Electrophoresis and Isoelectric Focusing. Electrophoresis was performed by the method of Hyden and Langer (1971) using 5% acrylamide in the running gel at pH 9 and 2.5% in the stacking gel at pH 7.5. All solutions contained 0.1% Triton X-100; 4 mA/tube was applied. The gels were stained with Amido-Schwartz and scanned by a laser densitometer. The method of Huang et al. (1973) was used for isoelectric focusing except that the acrylamide solution contained 0.2% Triton X-100 and the sample was overlaid with 30 μL of 2% Ampholine (pH 3.5–10 range) in 5% sucrose. The gels were sliced into 1-mm sections and incubated at 60 °C for 3 h in 0.5 mL of Soluene-100, and their radioactive content was measured by scintillation counting.

Other Assays. Protein was determined by the method of Lowry et al. (1951), or in the case of particulate samples, a modification of it (Lees and Paxman, 1972), with bovine serum albumin as standard. The presence of Triton X-100 or Emulphogene BC-720 caused the formation of a precipitate during the reaction; it was shown that this precipitate, and its removal by centrifugation, did not affect the final result. A microversion, in which a relatively large sample volume (100 μL) was used together with small volumes of proportionately more concentrated reagents (final volume, 210 μL), gave proportional results in the range 2–50 μg/mL. The method of Ellman et al. (1961) was used for the assay of AChE, adapted where necessary to a semi-micro scale, to permit measurement in a final volume of 250 μL. Eel AChE, freshly diluted in 0.2% Triton X-100, was used as a standard.

Results

Properties of ^{3}H-Labeled Triacetyl-BuTX. A ^{3}H-labeled monoacetylated BuTX was previously prepared for studies of ACh.R in muscle (Barnard et al., 1971; Chiu et al., 1974). A triacetylated species was prepared and used here, however, since this provided increased sensitivity for the assay of solubilized ACh.R. Two slightly overlapping peaks of labeled toxin, each with 3.0 [^{3}H]acetyl groups per molecule, were eluted in chromatography on CM-52 (see Materials and Methods). These appear to correspond to the two isomeric triacetylated forms recognized (but not separated) by Chang et al. (1973). The second (A, less acidic) of these peaks was used in the present studies. A fuller characterization of the species used will be given elsewhere. The specificity and irreversibility, at the endplate receptors, of the triacetyl-BuTX derivative used have been demonstrated by Dolly et al. (1977a).

Assay of ACh.R Activity. Initially a number of different types of assays was tested for routine quantitation of ACh.R activity. An ultrafiltration method employing Pellicon or Amicon membranes was found to be unsuitable due to the high amounts of free [^{3}H]BuTX that were retained on these filters, even after exhaustive washing with appropriate media. Likewise, the Millipore filtration assay described by Olsen et al. (1972) for electroplaque receptors could not be used because the muscle ACh.R–[^{3}H]BuTX complex was not retained by the membranes. A gel filtration method employing small columns (1 × 30 cm) of Sepharose 6B equilibrated with 0.2% Triton X-100/10 mM potassium phosphate buffer, pH 8.0, was used to separate free [^{3}H]BuTX from ACh.R–[^{3}H]BuTX. This allowed measurement of the labeled complex, but that assay was laborious and not very sensitive. Furthermore, it proved difficult to achieve, on the small columns needed for rapidity, a complete separation of the [^{3}H]BuTX–receptor complex from all of the free [^{3}H]BuTX. For those reasons, the DEAE-cellulose disc assay method was used; the total radioactivity bound to the DEAE-cellulose disc was directly proportional to the amount of ACh.R present in the reaction mixture over a wide concentration range. It is important to check that the ion-exchange capacity of the paper is not exceeded. The control values are very low, provided that the washing procedure described is used. The assay was shown to be specific, in that preincubation of the ACh.R preparation with a saturating concentration of unlabeled BuTX or α-neurotoxin (from *Naja naja siamensis* venom) completely blocked the binding of [^{3}H]BuTX as seen in this assay. The addition of cholinergic ligands such as d-TC or ACh at 10^{-4} M concentration to the reaction mixture inhibited the uptake of labeled toxin to the extent of at least 90%, even in crude extracts (Dolly and Barnard, 1974, 1975). The assay is rapid, convenient, and sensitive; the measured activities agree closely with those obtained using the gel-filtration method.

Solubilization of the ACh.R. The concentration of the ACh.R in innervated skeletal muscle is very low relative to that in fish electric organs. Chronically denervated muscle was, therefore, employed for the purification studies, since denervation produces a large increase in the number of [^{3}H]BuTX binding sites which show many of the properties of the receptor from innervated muscle (Barnard et al., 1975). The extent of this increase, at its maximum, varies with the muscle type (Table I). The mixed muscles of the cat leg supplied by the sciatic nerve proved a useful source of relatively high receptor content after 4 weeks of denervation.

Triton X-100 and Emulphogene BC-720 solutions were effective in solubilizing the active receptor; the latter was less effective but offered the advantage of transparency at 280 nm for direct protein measurement by absorbance. The total

TABLE I: Content of ACh.R in Denervated Muscles.[a]

Muscle	Days of denervation	nmol/kg wet weight	Content of denervated rel to innervated
Rat diaphragm	29	99	33
Mouse diaphragm	8	14	7
Rat soleus	8	108	14
Cat lower leg mixed muscles	28	88	44

[a] Receptor activity, expressed as nanomoles of the component that irreversibly binds labeled BuTX, measured in crude extracts in media containing Triton X-100. This represents (see text) an extraction of ~80% of the total amount of ACh.R present in the intact muscle.

TABLE II: Extraction of ACh.R. from Denervated Muscle.[a]

Length of extraction (h)	ACh.R. act. (pmol/mL)	Protein (mg/mL)	Spec act. (nmol/g of protein)
0.5	2.0	4.2	0.5
1	4.6	5.0	0.9
2	9.8	7.5	1.3
4	11.6	7.7	1.5
8	9.4	8.6	1.1
12	7.6	8.7	0.9

[a] The muscle preparations were extracted (1 g per 2.5 mL of medium) with 1.5% Triton X-100/50 mM potassium phosphate buffer, pH 8, in a shaking water bath at 4 °C for the periods stated. After centrifugation, the soluble extracts were assayed for [^{3}H]BuTX-binding activity and protein (see Materials and Methods).

content of ACh.R in a given muscle was determined by labeling it with [^{3}H]BuTX in the muscle itself to saturation of the receptor sites, washing, extracting the complex with Triton X-100 until no more radioactivity came into the solution (as described in Materials and Methods), and gel-filtering the extract on a column of Sepharose to determine the actual amount of complex present. As has been shown for the complex (Chiu et al., 1973), the maximum effect of Triton X-100 extraction of free receptor (at 4 °C) was reached at 1.5% concentration. The release of receptor from denervated cat leg muscle (cut up finely, but not homogenized) reached a maximum (about 50% of the receptor content of the muscle) in this medium at 4–6 h, thereafter declining (Table II). The extraction of protein, however, was close to its maximum at that period. The later decline of receptor activity is due to proteolytic degradation, since combined addition to the extraction medium of $PhCH_2SO_2F$ (10^{-4} M), benzethonium chloride (10^{-4} M), 0.1% oxanilic acid, and EDTA (10^{-3} M), as protease inhibitors, prevented this effect and allowed the extraction period to be extended to 15 h, considerably improving the yield of extracted receptor. A preliminary extraction of the chopped tissue in detergent-free buffer removed considerable protein and no receptor activity; this was, therefore, used in the purification scheme. As seen in Table III (fraction II), the specific activity in the extract in such a case was at least 2.4 nmol/g of protein, instead of the previous maximum of 1.5; in other preparations this value reached 2.9 nmol/g. In these conditions, the yield of receptor in the extract relative to the extractable receptor total in the muscle was 75–85%, after a single 15-h extraction as regularly used; the remainder could be removed in repeated extractions of the sediment, but this was not routinely done.

Purification of the Receptor. Gel filtration of the extract

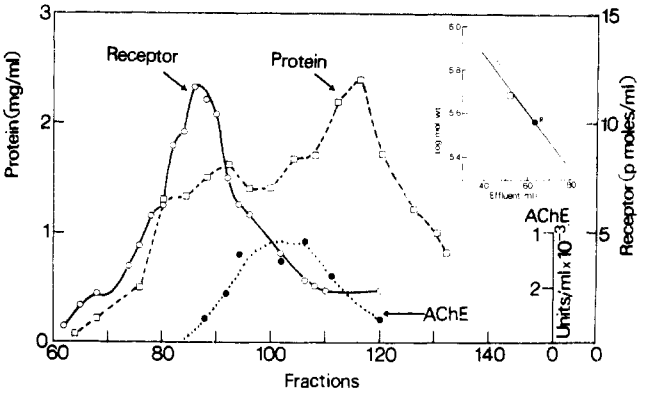

FIGURE 1: Gel filtration of a Triton X-100 extract (250 mL) of cat denervated leg muscles on a column (60 × 9.5 cm) of Ultrogel Ac22, equilibrated in 10 mM potassium phosphate buffer (pH 7.0)/0.2% Triton X-100/10^{-4} M EDTA (deoxygenated by bubbling with oxygen-free N_2). ACh.R activity (○), AChE activity (●), and protein (□) were measured in the fractions (30 mL). Insert: Gel filtration of a Triton X-100 extract of cat denervated leg muscles and protein markers on a column of Sepharose 6B (90 × 1.5 cm), equilibrated with 0.2% Triton X-100/10 mM potassium phosphate buffer, pH 8.0. A plot of the elution volume of thyroglobulin (△), urease (□), catalase (○), and the ACh.R (●, R) against log of the molecular weight is shown.

on an Ultrogel AcA 22 column gave a major peak of ACh.R activity, separated from most of the protein present (Figure 1). There was, as shown, very little AChE activity in the entire profile and the main receptor fraction obtained contained only a trace of this. (The same was also true when $PhCH_2SO_2F$, which is an inhibitor of some forms of AChE, was omitted from the initial extraction medium.) The gel filtration step gave a 3- to 4-fold purification; 50–60% of the applied receptor was pooled from the central fractions of its peak and concentrated. The concentrate was fractionated on an affinity column containing an immobilized cholinergic ligand (see Materials and Methods). At least 90% of the ACh.R activity applied became adsorbed to the resin, while the great bulk of the protein applied appeared in the break-through or after further washing with 0.2% Triton X-100/50 mM potassium phosphate buffer, pH 8.0 (Figures 2 and 3). In the first method tried, elution was with a linear gradient of NaCl; a major peak of ACh.R activity was then separated from a large protein peak (Figure 2). To facilitate assay of the effluent, the salt was removed by passage through a hollow-fiber dialyzer. In purification by this method, receptor of, e.g., initial specific activity of 8 nmol/g of protein, was applied, and the NaCl-eluted peak (Figure 2) contained 0.2 mg of protein and 28% of the applied ACh.R activity, at a specific activity of 155 nmol/g of protein. The same extraction and purification treatment was applied also to the ACh.R of denervated rat diaphragm and a specific activity of 250 nmol/g of protein was obtained.

In the second method used, the eluting NaCl was replaced by a cholinergic ligand. The antagonist, Flaxedil, was found to be much more effective than the agonists carbamoylcholine or decamethonium. As shown in Figure 3, 2.1 mM Flaxedil gave a specific displacement of the receptor from the affinity column, after almost all other protein had been removed by salt washes. A gradient in Flaxedil gave no improvement over the frontal displacement. The affinity chromatography gave on average about a 200-fold purification in that step (Table III). This contrasts with about 20-fold with the NaCl gradient solution. The Flaxedil method was used in all further purification of the receptor.

Final separation of the receptor from the contaminating protein still present was made by ion-exchange chromatog-

TABLE III: Purification of ACh.R from Cat Denervated Muscle.[a]

Fraction	Receptor act. (pmol)	Protein (mg)	Spec act. (nmol/g of protein)	Purification (fold)	Yield (%)
(I) Intact muscle	4128	3488	1.2 (0.066/g of tissue)	1	100
(II) Triton X-100 extract	3302	1395	2.4	2	80
(III) Ultrogel peak	1683	214	7.9	6.6	41
(IV) Affinity column					
total	855				21
fraction taken	493	0.36	1369	1141	12
(V) DEAE-Sephadex					
total	257				6.2
fraction taken	129	0.045	2867	2389	3.1

[a] The values given are from one preparation where all data were available. Other preparations could give higher final specific activities (see text.)

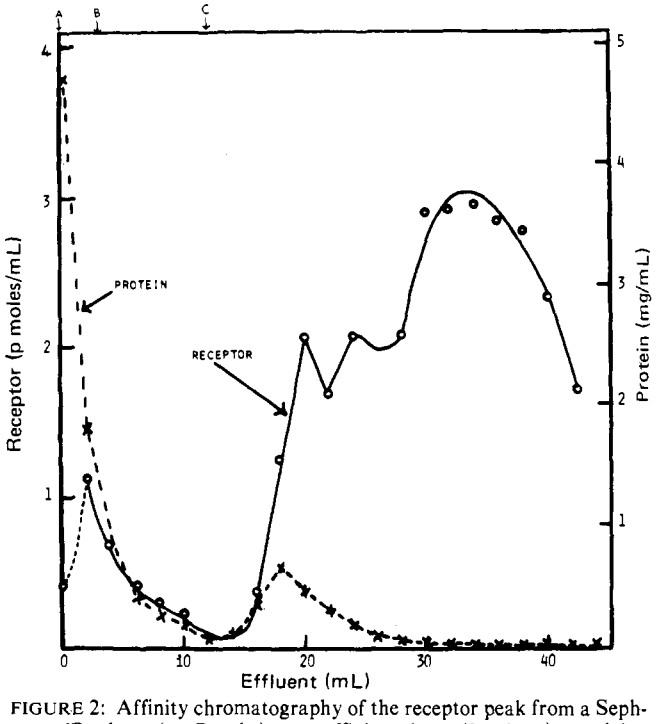

FIGURE 2: Affinity chromatography of the receptor peak from a Sepharose 6B column (see Results) on an affinity column (2 × 1 cm) containing a covalently attached quaternary ligand (see Materials and Methods). After the sample was loaded onto the column in 10 mM potassium phosphate buffer (pH 8.0)/0.2% Triton X-100, the column was washed with 4 mL of the same buffer (A) followed by 10 mL of 50 mM potassium phosphate buffer (pH 8.0)/0.2% Triton X-100 (B) to elute loosely bound protein; the receptor was then eluted with a linear gradient (C) of NaCl (0–0.6 M) in the same buffer. ACh.R activity (○) and protein (X) were measured in the fractions (2 mL) after removal of the salt by "in-line" dialysis using hollow fibers.

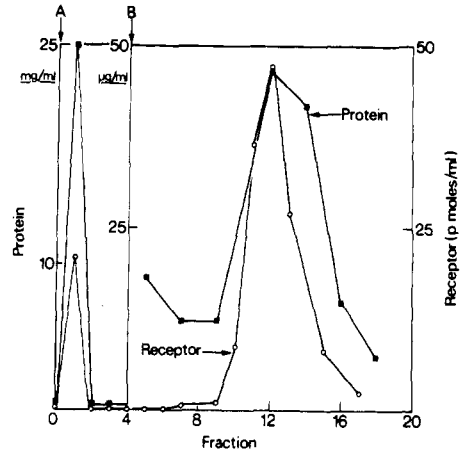

FIGURE 3: Affinity chromatography of the receptor peak taken from the Ultrogel column (Figure 1). The quaternary ligand affinity column (1 × 6 cm) was equilibrated with 50 mM potassium phosphate buffer (pH 8.0)/0.2% Triton X-100/10^{-4} M EDTA (equilibrating buffer). A concentrate of the receptor (5 mL) was loaded over a 1-h period and the column was then washed (at A) with 16 mL of 50 mM NaCl in equilibrating buffer. The receptor was then eluted (at B) with 2.1 M Flaxedil; in the latter medium, 1-mL fractions were collected with continuous in-line dialysis to remove the Flaxedil, as described in Materials and Methods. In fractions 0–4, the receptor activity (○) and protein content (■) are expressed as though concentrated to 1-mL fractions. Fraction 0 represents the breakthrough volume alone. Note the large change in protein scale from A to B.

raphy on DEAE-Sephadex. Almost all the receptor became bound, and after removal of extraneous protein by a dilute salt wash, a single peak of receptor was eluted by a gradient in NaCl (Table III). The central fractions of this peak yielded the pure receptor.

Criteria of Purity. In six different preparations, the final specific activities of the product were in the range 2000–6000 nmol of toxin bound/g of protein. The lower values were believed to be due to the presence of trace amounts of protease, as evidenced by their decrease on storage at 5 °C. Purity was assessed on the peak fraction in the final chromatogram, having 5000–6000 nmol/g, and this gave a single sharp band in gel electrophoresis in nondenaturing media (Figure 4). With specific activities lower than the latter value, trace contaminant bands were seen, but these were variable.

Polyacrylamide gel isolectric focusing of [^{3}H]BuTX–ACh.R complex, formed by incubation of the ACh.R with [^{3}H]BuTX under conditions that minimize proteolysis (see Materials and Methods) gave a single peak of radioactivity with an isoelectric point of 5.3. Trace amounts of AChE activity were detectable in the receptor peak from the affinity column, but were totally absent from the purified receptor fractions eluted from the DEAE-Sephadex column. On the assumption that muscle AChE has the same specific activity as the purified electroplaque enzyme (Rosenberry et al., 1972) and from the sensitivity of the assay, it can be calculated that the pure ACh.R protein has less than 0.0004% of active AChE. The molecular weight of the receptor was estimated only from its Stokes' radius in 0.2% Triton X-100 solution, as measured in gel filtration (Figure 1, insert). The purified receptor gave a peak in the same position as that in crude extract with apparent molecular weight, estimated as in Figure 1, of 370 000.

TABLE IV: Rates of Reaction of ACh Receptors with α-Neurotoxins.

Toxin	Receptor	Temp (°C)	k_1 (M^{-1} s^{-1})	Ref
[^{3}H]BuTX	Denervated cat muscle:	25		
	partially or fully purified		2.9×10^5	This work
	impure, aged [a]		1.0×10^5	This work
	membrane-bound		0.3×10^5	(Barnard et al., 1977)
[^{125}I]BuTX	Denervated rat muscle: partly purified	35	3.1×10^5 and 1.2×10^5	(Brockes & Hall, 1975)
	crude extract	21	0.9×10^5	(Colquhoun & Rang, 1976)
	membrane-bound		0.3×10^5	
	Rat brain: crude extract	RT	6.8×10^5	(Lowy et al., 1976)
	Rat superior cervical ganglion: crude extract		1.7×10^5	(Fumagalli et al., 1976)
Naja naja siamensis α-neurotoxin	Frog muscle		1.5×10^5 (blockade rate)	(Lester, 1972)

[a] Receptor solution not treated with protease inhibitors or deoxygenated; it was gel filtered, but not subjected to affinity chromatography, and kept at 4 °C for more than 1 day.

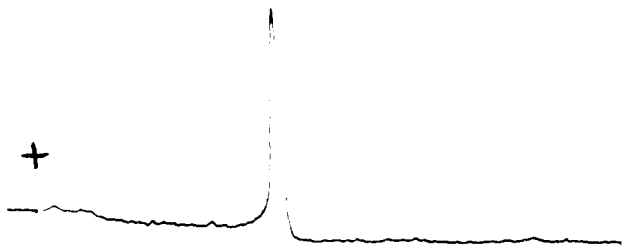

FIGURE 4: Scan of a polyacrylamide gel (5%) electrophoretogram of the purified ACh.R (see Results) run at pH 9, under nondenaturing conditions, and stained with Amido-Schwartz (see Materials and Methods). Migration is from the right.

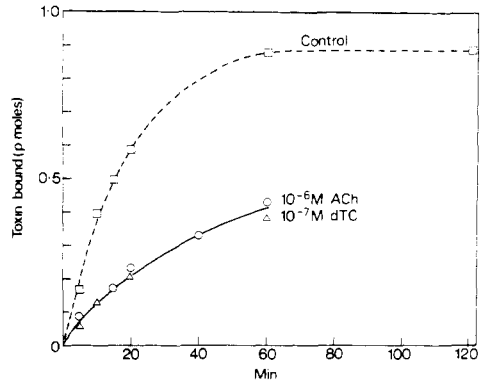

FIGURE 5: Reaction of pure ACh receptor with [^{3}H]BuTX, and the protections exerted by specific ligands. [^{3}H]BuTX (6 nM) was reacted with a fixed amount of receptor solution, and the amount bound per mL of solution (□) as a function of time was determined in a 100-μL aliquot by the DEAE-cellulose filter disc assay. The medium was 25 mM phosphate buffer, pH 8, in 0.2% Triton X-100, at 25 °C. The broken curve is theoretical, for second-order behavior. Parallel reactions conducted in the presence of 1.0×10^{-7} M d-TC (△) or 1.0×10^{-6} M ACh (○) are shown as examples of those used, in second-order plots, to determine the initial retardation in the series for K_P estimation. A 20-min preincubation with each ligand was given at 25 °C: in the case of ACh, 3×10^{-6} M DFP was added.

Reactions of ACh.R with BuTX and Cholinergic Ligands. When the binding of [^{3}H]BuTX to pure ACh.R was studied kinetically, regular second-order behavior was found (Figure 5). This gave a rate constant which is consistent with those reported for other forms of this receptor (Table IV). The same rate constant was obtained for the crude and purified ACh.R. When the preparation was not guarded from air oxidation and proteolysis the rates became slower and biphasic. Brockes and Hall (1975) found a fast (30% of the total) and a slow (70% of the total) component in [^{125}I]BuTX reaction of ACh.R in partially purified preparations from rat muscle. The rate of reaction of [^{3}H]BuTX and ACh.R was greatly accelerated by increasing the temperature up to 37 °C, but at higher temperatures the receptor (tested when partially purified) was rapidly inactivated. Essentially the same reaction rate at 25 °C was obtained over the pH range 6.5–8.0.

When the reaction of ACh.R with [^{3}H]BuTX was performed in the presence of a cholinergic ligand, the reaction was, as predicted, strongly retarded. Saturating concentrations of ligands (d-TC, ACh, or decamethonium), i.e., in the range 10^{-5} to 10^{-4} M, protected 95–100% of the BuTX binding sites. At the lower toxin concentrations used the initial velocity could be measured (Figure 5), so it was possible to estimate the protection constants (K_p) using the equation (Mildvan and Leigh, 1964; Meunier et al., 1972)

$$\frac{v_i}{v} = \frac{K_p}{K_p + [L]}$$

where v and v_i are the initial rates measured respectively in the absence and presence of ligand, L. The protection constants obtained for ACh, carbamoylcholine, decamethonium, d-TC, and hexamethonium were 1.0×10^{-6}, 1.0×10^{-5}, 0.5×10^{-6}, 0.5×10^{-7}, and 3.4×10^{-4} M, respectively. These measurements were made under conditions where the receptor was preequilibrated with the ligand prior to the addition of the [^{3}H]BuTX. However, identical K_p values were obtained when agonist and toxin were added simultaneously, suggesting that no desensitization was occurring. The K_p values obtained are in close agreement with the known pharmacological effectiveness of the ligands on skeletal muscle, and fairly similar to those for muscle synaptic membranes (Barnard et al., 1977). The muscarinic ligands atropine and pilocarpine, on the other hand, showed no affinity for ACh.R at concentrations of 10^{-5} and 5×10^{-6} M, respectively.

Discussion

Despite the very low amounts of ACh.R present in mammalian skeletal muscle, and also the inaccessibility of large quantities of denervated muscle, it has been possible to solubilize the receptor with nonionic detergents and purify it to apparent homogeneity using gel filtration, affinity, and ion-

exchange chromatography. It is very difficult to achieve a good purification of such membrane-bound proteins due to the many similarities they exhibit, e.g., in molecular weight and isoelectric point. Therefore, attempts to use chromatography on hydroxylapatite and DEAE-resins in the purification procedure were unsuccessful, only about a 2-fold purification being achieved, while the yields were very low. Although gel filtration on Ultrogel AcA22 did not give a very high purification (3–4-fold), it did afford separation of most of the AChE activity. Affinity chromatography on a quaternary ammonium ligand, covalently linked to Sepharose 4B gel, with biospecific elution by Flaxedil, gave an excellent purification, although the yield was rather low. The low recovery of receptor may be due to the high levels of protease activity characteristic of denervated muscle (McLaughlin and Bosmann, 1976). Recent experiments have shown that, unless several protease inhibitors (see Materials and Methods) are present throughout, the receptor becomes proteolyzed to an inactive form with a lower molecular weight, that can be separated by chromatography on Sepharose 6B (J. O. Dolly, R. Shorr, and E. A. Barnard, unpublished). The proteolyzed form has a higher isoelectric point. In the purification of the ACh.R from innervated and denervated rat diaphragm, using a cobra toxin affinity resin, Brockes and Hall (1975) obtained much lower specific activities (190 and 530 nmol/g of protein, respectively) but reasonable yields (20%). We have also employed an affinity column containing a bound α-neurotoxin together with elution by carbamoylcholine; this gave a purification better than that reported here (Dolly et al., 1977b). Recently, Almon and Appel (1976a) reported that a high degree of purification of the ACh.R from innervated and denervated rat muscle can be achieved by affinity chromatography on a Sepharose–concanavalin A resin. However, the final specific activities of the purified material could not be measured because of insufficient amounts of protein.

The maximum specific activity that we obtain is about the average of those that have been reported for this receptor from *Torpedo* (range 2000–12 000 nmol/g) and *Electrophorus* (4000–11 000), as reviewed by Changeux (1975) and Karlin et al. (1975), but values up to 12 000 nmol/g have been reported in only one or two of these cases. A factor, other than residual impurity, that may decrease our value below the true one for the muscle receptor is that the Lowry method of protein measurement used here with serum albumin as standard has been reported to give a lower specific activity for *Torpedo* receptor than that found on the basis of amino acid analysis (Eldefrawi et al., 1975).

The purified receptor appears to be homogeneous on polyacrylamide gel electrophoresis under native conditions. The amino acid composition of an acid hydrolysate of the same material confirmed its protein nature. The receptor BuTX complexes that were formed with crude and pure receptor showed identical isoelectric points, suggesting that no appreciable proteolysis has occurred during the isolation period. The apparent molecular weight of 370 000, obtained by gel filtration in 0.2% Triton X-100, is probably an overestimate due to bound detergent. A similar value was found by such a gel filtration for the electroplaque receptors, and a corrected figure of 250 000 (Hucho and Changeux, 1973) or 330 000 (Edelstein et al., 1975) was obtained by other means. The apparent molecular weight obtained similarly for innervated muscle receptor in crude extracts was the same as for denervated. The size of the subunit of the pure muscle ACh.R, as seen in dodecyl sulfate electrophoresis, will be documented elsewhere.

The purified protein exhibited many of the properties of the ACh.R in situ; for example, the association rate constant for binding of [^{3}H]BuTX was similar to that determined for blockade of frog muscle by α-neurotoxin (Table IV). The affinity constants obtained for a series of cholinergic agonists and antagonists for the purified and membrane-bound (Barnard et al., 1977) receptor were similar. These affinity constants are consistent with the pharmacological potencies of these ligands; the nonnicotinic ligands pilocarpine and atropine showed very low affinity ($K_P > 10^{-4}$ M) for the receptor. No evidence of desensitization of the receptor by the ligands was obtained, in the case of this soluble receptor; that is to say, identical K_p values were seen with and without preincubation of the receptor with the ligand. It should be noted that desensitization, although found with *Torpedo* membrane fragments, has been found absent in Triton-solubilized or purified *Torpedo* receptor (Weber et al., 1975; Sugiyama and Changeux, 1975; Weiland et al., 1976).

Although denervated and innervated muscle receptors are very similar in their molecular weights (Chiu et al., 1973), their rates of toxin binding and their interaction with concanavalin A (Brockes and Hall, 1975; Almon and Appel, 1976a) some differences between them have been reported. For example, denervated muscle receptors appeared to have a lower affinity for d-TC than those in innervated muscle in situ (Beranek and Vyskocil, 1967; Lapa et al., 1974) or in extracts (Brockes and Hall, 1975), but this difference could not be detected by others (Jenkinson, 1960; Alper et al., 1974; Almon and Appel, 1976b; Colquhoun and Rang, 1976). The latter disagreement in results may reflect various experimental errors present in the measurements on extracts (see Colquhoun and Rang, 1976) or in differences in the electrophysiological systems used for the measurements in situ (see Dolly et al., 1977a). The rates of turnover of the two forms are different (Berg and Hall, 1974; Devreotes and Fambrough, 1975; Chang and Huang, 1975; Merlie et al., 1976); this need not signify they are different proteins but could be due to a single protein, differently arranged in the junctional and extra-junctional membranes, or influenced by the different metabolic state of denervated muscle (McLaughlin and Bosmann, 1976). Moreover, the receptor turnover rate varies with the muscle electrical activity (Hogan et al., 1976; Shainberg and Burstein, 1976). A difference, of 0.15 of a pH unit, was observed in the isoelectric points of the two receptors (Brockes and Hall, 1975), but, again, it has been suggested by Changeux and Danchin (1976) that this arises from a posttranslational modification of a single protein type. Moreover, the latter possibility might explain the observed differences (Neher and Sakmann, 1976; Dolly et al., 1977a) in the ion channel associated with ACh.R. in synaptic and extra-synaptic regions of muscle. The position is still uncertain, however, as to whether there is any difference between these two receptor types. The characterization of the denervated muscle receptor should be helpful in deciphering the molecular constitution of the endplate receptor.

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