BIOCHEMICAL STUDIES ON TORPEDO CALIFORNICA ACETYLCHOLINE RECEPTORS*

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INTRODUCTION

The isolation and biochemical or biophysical characterization of cholinergic receptors depends on a reliable assay of receptor function and on a source of large quantities of such material. We have recently developed a simple assay (1) for detection and determination of acetylcholine receptors in electroplax membranes and in detergent-solubilized membrane preparations. This assay is based on the well established specific and essentially irreversible association of ^{125}I-labeled α-bungarotoxin (^{125}I-α-Bgt) with cholinergic receptor and utilizes the differing isoelectric points of the free toxin (~9.2) and the toxin-receptor complex (~5) (2). Using this assay it can be shown that the electric tissue of Torpedo californica contains 0.5 to 1.0 nmoles of bungarotoxin binding sites per gram tissue. This is clearly a tissue of choice for biochemical characterization of acetylcholine receptors and for isolation of large quantities of these molecules. In this article we discuss briefly the results of our recent studies of the isolation and characterization of acetylcholine receptors from this tissue. The details are described elsewhere (2–9).

ELECTROPLAX MEMBRANES

When Torpedo californica electroplax membrane preparations are centrifuged in sucrose density gradients and the profiles for acetylcholine esterase, ATPase, α-bungarotoxin binding, and total protein determined, the results shown in Fig. 1 are obtained. The most interesting feature is a clear separation between particles which contain acetylcholine receptor and acetylcholine esterase activity. It is also evident that the receptor and esterase are associated with different membrane structures or particles, and secondly that they are different molecules rather than different binding sites on one molecule. Much of the esterase activity has been lost during the membrane preparation due to its solubility in aqueous salt solution; however, preparations made by homogenization of tissues in sucrose solution containing very low salt concentrations give re-

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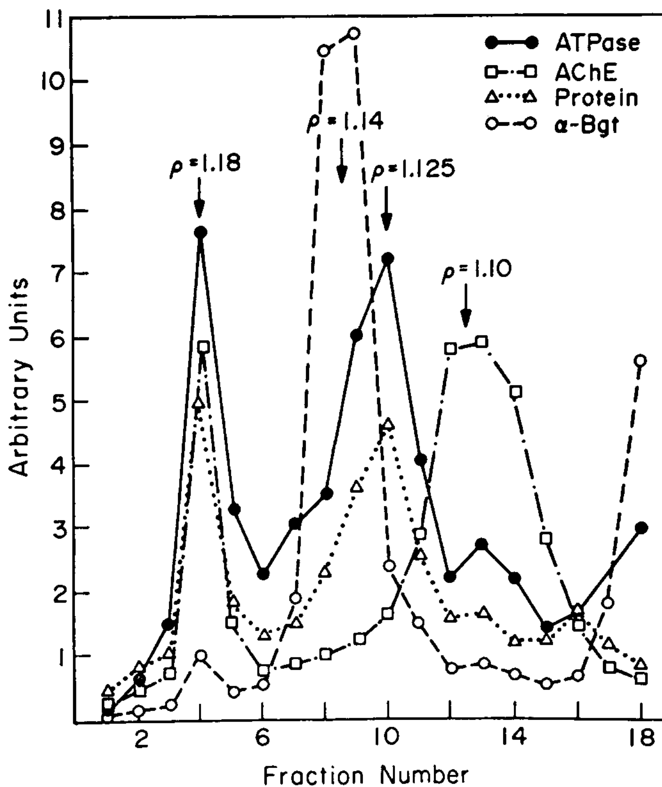

Fig. 1. Sucrose density gradient centrifugation of Torpedo californica electroplax membrane fragments. One arbitrary unit equals 0.24 μg ^{125}I-α-Bgt, 1.34 mg protein, 43 μmoles AcCh hydrolyzed per minute and 14 μmoles ATP hydrolyzed per hour.

sults similar to those shown. Fractions containing high acetylcholine receptor activity from sucrose density gradient centrifugations can be shown to contain ~ 5–10% of the total protein as receptor protein, assuming one α-Bgt site per 100,000 daltons of receptor protein.

SOLUBILIZATION OF ACETYLCHOLINE RECEPTOR

Several detergents were investigated to achieve solubilization of acetylcholine receptors from Torpedo membrane fragments. In Fig. 2 a comparison is made of the ^{125}I-α-Bgt binding activity and total membrane protein extracted, as a function of detergent concentration. Clearly, sodium dodecyl sulfate and cetyltrimethylammonium bromide are unsuitable, causing inactivation at low detergent concentration. Triton X–100 yielded extracts of greatest specific activity and it was found that this detergent in the concentration ranges shown, could extract 90–100% of the α-Bgt binding sites of membrane preparations. The extracted acetylcholine receptor was shown to be most likely a protein given its susceptibility to proteolytic hydrolysis with concomitant destruction of α-Bgt binding capability (3). When chromatographed on columns of Sepharose 6B the receptor behaved as a macromolecule with approximately the same Stokes radius as β-galactosidase,

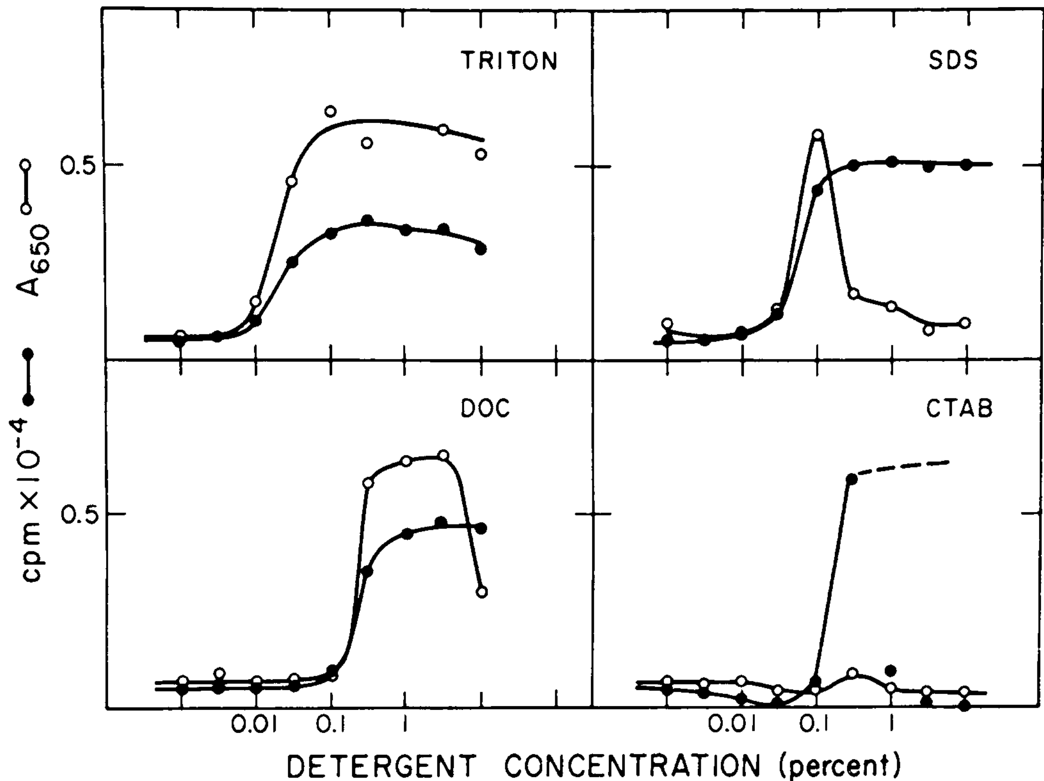

Fig. 2. Extraction of acetylcholine receptor from Torpedo membranes with various detergents.

and little purification was achieved. Similarly, on sucrose density gradients receptor and protein are only partially separated (Fig. 3). The isoelectric point of the α-Bgt receptor complex, as shown in Fig. 4, was approximately 5. Unfortunately most of the total protein in the extract appeared to have an isoelectric point of approximately the same value, and this finding precluded the use of most common procedures for the purification of the receptor.

AFFINITY CHROMATOGRAPHY

Quaternary ammonium substituted Sepharose 2B affinity resin with the ligand shown was synthesized $[-NH-(CH_2)_5CO-NH-(CH_2)_3-\overset{+}{N}(CH_3)_3\bar{X}]$. Chromatography of the Triton-extracted Torpedo membranes on this resin yielded the profile shown in Fig. 5. Most of the protein was eluted at the void volume and a sodium chloride gradient was used to elute toxin binding activity and acetylcholine esterase activity, as shown. This clear separation of α-Bgt binding and acetylcholine esterase activity again shows separation between the acetylcholine receptor and acetylcholine esterase, as discussed earlier for the results obtained with membrane fragments. The receptor preparation obtained from affinity chromatography was found to bind one α-Bgt molecule (8000 MW) per 130,000 daltons of protein, and the yield of this material was approximately 100 mg per kg of

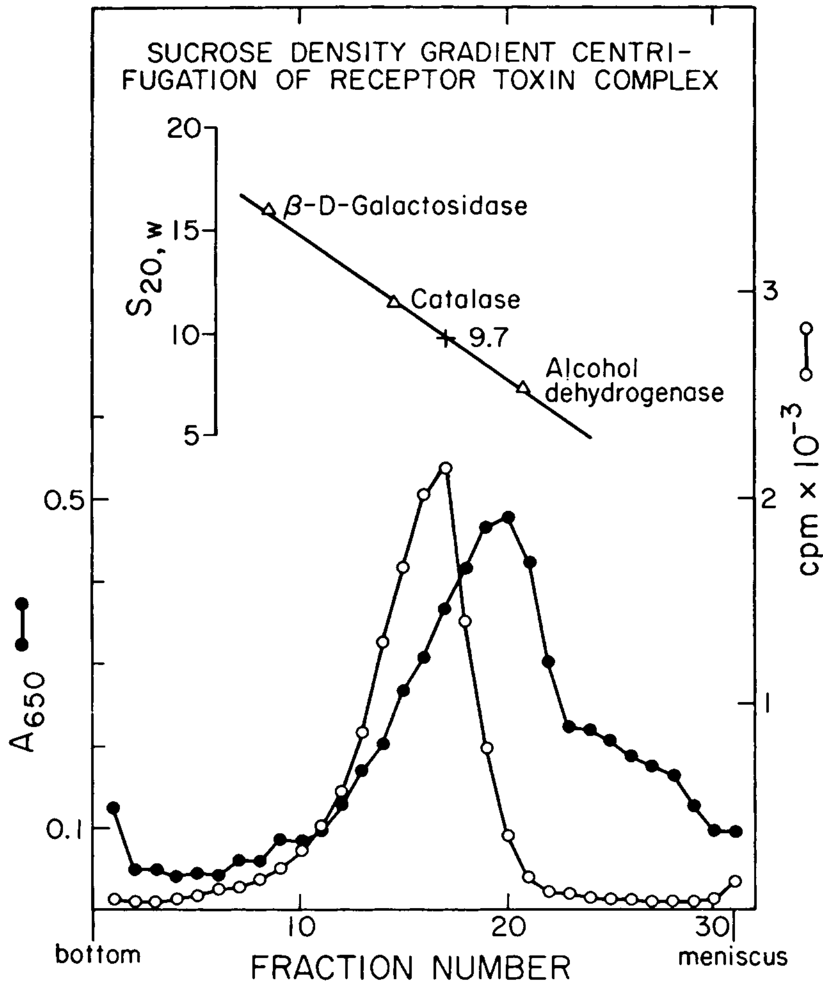

Fig. 3. Sucrose density gradient centrifugation of ^{125}I-α-Bgt-AcChR complex.

electric tissue (8). Some further purification was achieved by DEAE cellulose chromatography following concentration on an Amicon Diaflo ultrafiltration apparatus using a PM-10 membrane. The material following DEAE cellulose chromatography bound, in our best preparation, one α-Bgt molecule per 95,000 daltons of protein. The overall purification achieved corresponds to a factor of about 65 fold from the membrane preparation or about 500 fold from the original tissue preparation.

PROPERTIES OF THE ISOLATED ACETYLCHOLINE RECEPTOR

The isolated material behaved as a high molecular weight component upon chromatography on Sepharose 6B yielding an apparent molecular weight of ~ 450,000–500,000. Sucrose density gradient centrifugation yielded an S value of 9.4 when compared to various protein standards. A similar discrepancy in apparent Stokes radius and

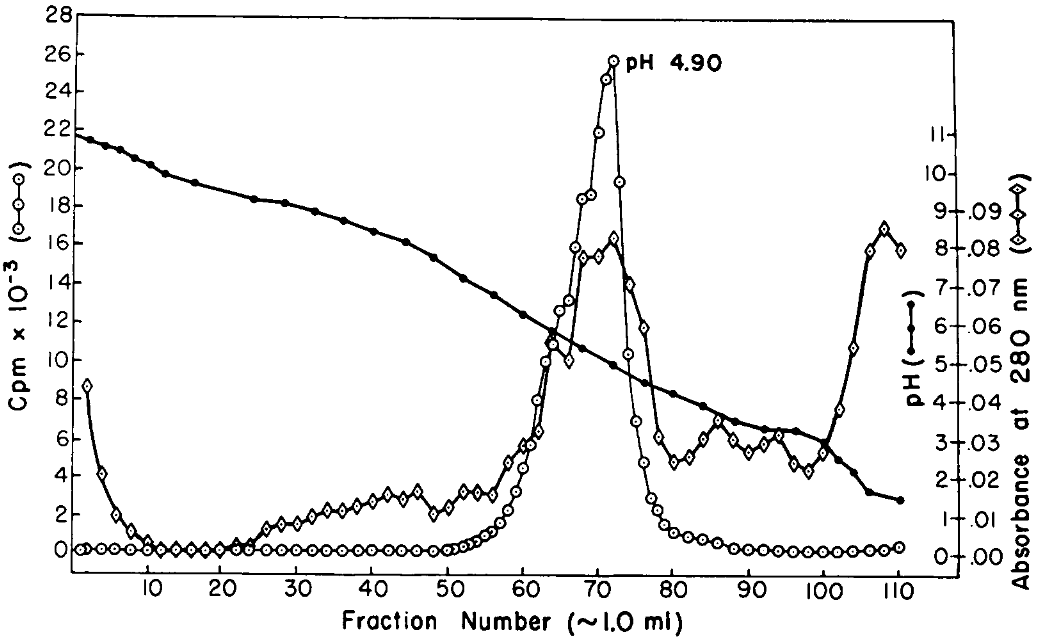

Fig. 4. Isoelectric focusing of solubilized AcChR as its ^{125}I-α-Bgt complex.

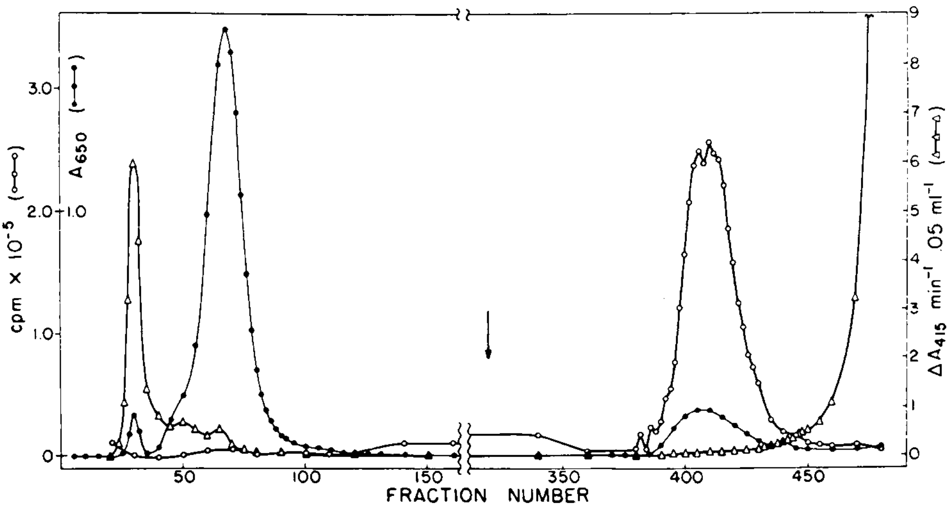

Fig. 5. Affinity chromatography of solubilized AcChR on Sepharose–2B affinity resin. Protein (●–●), AcChE (△–△), AcChR (○–○). Sample in 10 mM sodium phosphate, pH 7.4. A linear salt gradient, to 0.5 M NaCl, was started at fraction 320.

S value has been reported for α-Bgt binding components in detergent extracts of Electrophorus electricus electroplax membranes (2, 10). Further studies have shown that the isolated receptor is a glycoprotein containing ~ 7% total neutral sugars; mannose, galactose, and N-acetyl-D-glucosamine have been tentatively identified. Tests for sialic acid were negative. The glycoprotein nature of the receptor has also been demonstrated by acrylamide gel electrophoresis in SDS; staining for protein with Coomassie Blue and for carbohydrate with a periodic acid-Schiff reagent demonstrates coincidence of the protein and sugar stains. Two major and two minor polypeptide chains have been detected with molecular weights in the neighborhood of 40,000, the higher molecular weight polypeptides containing more sugar-staining material than the lower molecular weight ones. At the present time it is not known whether the multiplicity of polypeptide bands obtained in the SDS gel pattern is due to different polypeptide chains or to microheterogeneity due to the glycoprotein nature of the receptor subunits. In any case, it appears that in the preparation described, one α-Bgt molecule binds per approximately two receptor subunits. It is conceivable that the acetylcholine receptor is composed of dissimilar subunits, with binding site and ion channel located on distinct polypeptide chains.

BINDING OF CHOLINERGIC LIGANDS

Identification of isolated acetylcholine receptors requires an analysis of their affinities for acetylcholine and other cholinergic effectors. We have used several methods for such studies including equilibrium dialysis, inhibition of α-Bgt binding, and fluorescence quenching of a specific dye. Equilibrium dialysis data obtained for the binding of acetylcholine to the isolated receptor are presented in Fig. 6. One type of binding site was detected with a dissociation constant of 2.3×10^{-6} M. Similar results obtained for d-tubocurarine and decamethonium are presented in Table I. As indicated, the number of binding sites for these three ligands is approximately equal to half the number of α-Bgt binding sites at the pH used. Table I also indicates the noncooperative nature of the binding of acetylcholine and other cholinergic ligands.

The rate of ^{125}I-α-Bgt binding to the acetylcholine receptor is slowed down by cholinergic compounds and half maximal inhibition of toxin binding is achieved at differing concentrations for each ligand. Table II lists the potency of a variety of drugs in this respect compared to acetylcholine. It is clear that nicotinic agents such as tubocurarine, flaxedil, and nicotine are more powerful inhibitors of toxin binding than are the muscarinic drugs atropine and pilocarpine or the anti-acetylcholine esterase eserine. All of the binding data so far clearly indicate the nicotinic nature of the purified acetylcholine receptor from Torpedo californica electroplax.

A potent inhibitor of acetylcholinesterase (11), bis (3-aminopyridinium-1, 10 decane) (DAP), has, in our hands, also proven to be a good ligand for the acetylcholine receptor from Torpedo californica. The intense fluorescence of DAP at 400 nm, when excited at 330 nm, is strongly quenched upon binding to the receptor. The fluorescence quenching, which occurs as a consequence of the formation of a receptor–DAP complex, can be quantitated to determine the affinity of the receptor for the ligand.

No evidence of cooperativity could be detected in the resulting linear graphs of the Klotz treatment of the data (12). Pharmacological agonists and antagonists of acetylcholine were inhibitory of DAP–receptor complex formation in a competitive fashion with linear Klotz plots (Table III). The dissociation constants shown in Table III are lower in several

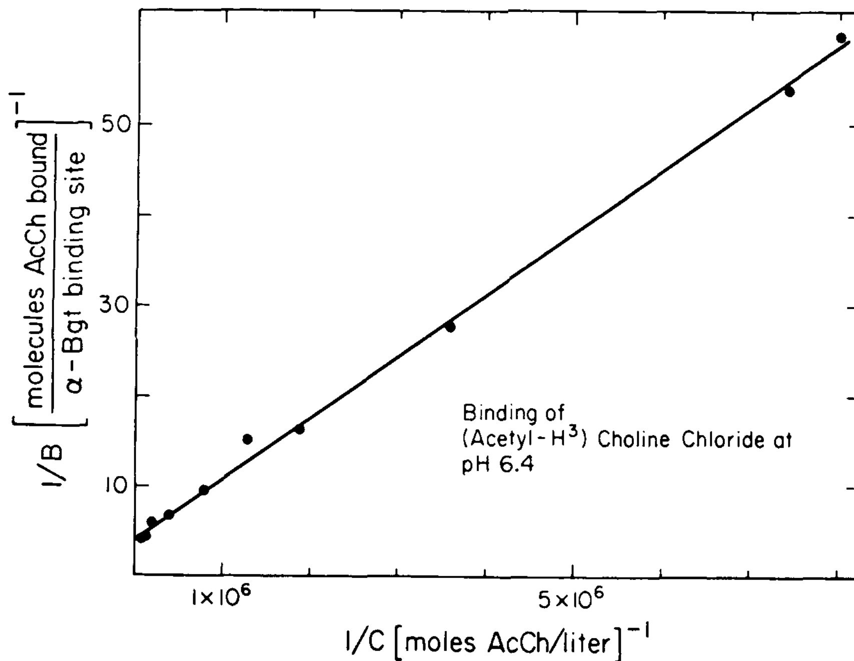

Fig. 6. Double reciprocal plot of AcCh binding to purified AcChR at pH 6.4. Equilibrium dialysis was carried out as described in Methods. Ligand concentrations from 0.01 to 1.0 × 10^{-5} M are shown. The ordinate is expressed as molecules of ligand bound per Bgt site. See DISCUSSION for pH effects.

Table I. Ligand Binding Parameters[a]

Ligand	K_D	B max / B Bgt	n (Hill coefficient)
AcCh	2.3 ± .3 μM	.42 ± .03	.93 ± .03
d–TC	6.4 ± .5 μM	.52 ± .05	1.05 ± .10
Deca	55 ± 15 μM	.41 ± .15	1.08 ± .03

[a]Binding of AcCh, d–TC and Deca were studied by equilibrium dialysis. The values presented are those obtained by equilibrium dialysis at pH 7.2, 200 mM NaCl.

Table II. Drug Profile of Purified AcChR[a]

Ligand	Log equipotent molar ratio relative to AcCh
AcCh	0.00
d–TC	0.45
Flaxedil	0.90
Nicotine	1.55
Deca	1.75
Carbamylcholine	1.75
Hexamethonium	2.0
Atropine	2.5
Choline	3.2
Eserine	3.3
Pilocarpine	3.5

[a]Average values from 2 to 6 experiments for each ligand are given.

Table III. Binding of Cholinergic Fluorescent Probe[a]

Compound	Dissociation constant M
DAP[b]	1.3 (± 0.2) × 10^{-7} [c]
AcCh	5 (± 1) × 10^{-6}
d–TC	1.8 (± 0.2) × 10^{-7}
Deca	2.5 (± 0.2) × 10^{-6}
Carbamylcholine	8 (± 1.5) × 10^{-5}

[a]In 10 mM sodium phosphate, pH 7.4.

[b]Gift of Dr. D. Sigman.

[c]α-Bgt prevents binding of DAP.

instances than those presented in Table I; this is due to an appreciable effect of ionic strength.

The noncooperativity of ligand binding observed with the purified receptor is of interest because it has been shown that conductance changes in the postsynaptic membrane occur in a cooperative fashion as a function of drug concentration (13). We consider it unlikely that the noncooperativity of ligand is a result of extraction of the receptor from the membrane since Torpedo californica electroplax membrane preparations bind decamethonium in a similar noncooperative fashion. It is clear therefore that cooperative effects must occur somewhere between binding of the cholinergic agonist and the opening of ion channels in the membrane in vivo.

CONCLUSION

It is now possible to make the statement that a neurotransmitter receptor, specifically that for acetylcholine, has been isolated in functional form, at least as regards transmitter binding properties. This receptor is a membrane-bound glycoprotein and may be classified as a nicotinic acetylcholine receptor. It now becomes possible to conduct biochemical and biophysical characterization of the isolated material at the molecular level, something that is difficult to do while the receptor is still associated with membrane particles or in vivo. Since preliminary results indicate that it is feasible to reconstitute the isolated receptor with phospholipids, it should be possible to conduct similar biochemical and biophysical studies, still at the molecular level, with the system at least partially reconstituted.

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