Binding of Acetylcholine and Related Compounds to Purified Acetylcholine Receptor from Torpedo Californica Electroplax.[1]

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ABSTRACT: The binding properties of the purified acetylcholine receptor from Torpedo californica were investigated. One type of binding was observed for acetylcholine (K_D = 2.3 μM), dimethyl tubocurarine (K_D = 6.2 μM), and decamethonium (K_D = 55 μM). No cooperativity was observed in ligand binding. By virtue of its ligand binding properties, the purified receptor is nicotinic in nature.

Introduction:

Binding of AcCh* and various cholinomimetic drugs to particulate fractions from the electric organ of Torpedo marmorata has been studied extensively (for a review see Ref. 1), and solubilization of electroplax membrane preparations in nonionic detergents seems to have little effect on binding of snake venom toxins (2) and small ligands (2,3). Purification of neurotoxin-binding membrane components from the electric organs of Narcine entemedor (4), Torpedo marmorata (5),

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* Abbreviations used are: acetylcholine, AcCh; dimethyl-tubocurarine, d-TC; Decamethonium, Deca; α-Bungarotoxin, αBgt ; acetylcholine receptor, AcChR; acetylcholinesterase, AcChE; di-isopropylfluorophosphate, DIFP.

Electrophorus electricus (6, 7) and Torpedo californica (8) has recently been reported. However, identification of isolated acetylcholine receptors requires an analysis of their affinities for acetylcholine and other cholinergic effectors. In this communication we describe the binding of AcCh, dimethyl-tubocurarine and decamethonium to the purified α-bungarotoxin binding component from Torpedo californica electroplax (8); the inhibitory effect of a variety of drugs on binding of αBgt is also reported. The data indicate that the binding specificity of the purified material is that of a nicotinic AcChR.

Experimental:

Materials: DIFP was from Aldrich. All radioactive ligands were from Amersham-Searle; their activities were as follows: ^{14}C-d-TC 83 mCi/mM, 112 mCi/mM; ^{3}H-AcCh 290 mCi/mM; ^{3}H-Deca 400 mCi/mM; ^{14}C-Deca 15 mCi/mM. ^{125}I-αBgt was prepared as described previously (9) and had a specific activity of 1-3 x 10^6 cpm/nmole, depending on the age of the preparation. Dialysis tubing (Union Carbide) was extracted with boiling water before use.

Preparation of AcChR: αBgt-binding material was purified by affinity chromatography as described previously (8). The product was concentrated using a PM-10 membrane in an Amicon ultracentrifugation cell and then passed through a HAWP 0.45 μ Millipore filter to remove particulate matter. Residual AcChE in the preparation (amounting to $<1\%$ of the quantity extracted from the tissue as determined by the method of Ellman (9)) was inhibited by DIFP. Two moles of DIFP were added per mole of AcChR, and excess DIFP was removed by extensive dialysis against dilute buffer (10 mM sodium phosphate pH 7.2; 0.1% Triton X-100). This treatment did not result in loss of αBgt binding activity nor were Deca or d-TC binding affinities affected. The final product had a protein concentration of 1-6 mg/ml, as determined according to Lowry et al. (10) and a specific activity of 4-7

nmoles αBgt bound per mg of protein as measured by the DEAE-cellulose disk assay (11).

Equilibrium Dialysis: AcChR (0.2 ml containing approximately 2 nmoles of αBgt binding sites) was placed in 1 cm diameter dialysis tubing. Dialysis was carried out against 5 ml of 10 mM sodium phosphate, 200 mM sodium chloride, .1% Triton X-100, and the appropriate concentration of radioactive ligand. The medium had a pH of 7.2 (unless otherwise indicated). The bag and medium were gently agitated in 10-ml screw-cap vials on a horizontal rocker (Buchler instruments) for 16-20 hours at 4° C. AcChR concentration as measured by αBgt binding activity remained constant throughout the experiment, which indicates that neither receptor inactivation nor sample dilution occurred during dialysis. Substitution of a Ringer's formula (12) for sodium chloride did not alter binding properties. Duplicate 0.050-ml aliquots were removed from both inside and outside the bag after dialysis, solubilized in 0.5 ml NCS (Amersham), and counted in 8 ml of 0.4% Permablend TM-III (Packard) in toluene. Differences between sample and medium were taken to represent ligand bound to receptor, while calculation of ligand concentration was based on radioactivity in the outside medium. The amount of ^{3}H-acetate present in the external buffer, due to spontaneous hydrolysis of AcCh,was measured before and after dialysis by means of an assay based on the differential solubility of acetate and acetylcholine at low pH in a toluene-based scintillator cocktail (R. Russell, unpublished). Hydrolysis of AcCh during dialysis usually was less than 4%.

Toxin Binding Protection Studies: Purified DIFP-treated AcChR (approximately 1 nmole αBgt binding sites) was incubated with varying concentrations of ligand at room temperature for 30 minutes in 10 mM sodium phosphate, pH 7.4, and .1% Triton (total volume 0.120 ml). ^{125}I-αBgt was then added (ca. 2 nmoles in 0.005 ml). After a toxin exposure time of 60 seconds, 0.100 ml of the mixture was pipetted onto a disk of DEAE-cellulose; disks were washed and counted as described previously (11).

Table I. Ligand Binding Parameters

Ligand	K_D	$\frac{B\ max}{B\ Bgt}$	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

Binding of AcCh, d-TC and Deca was studied by equilibrium dialysis as described in Methods. The values presented are those obtained by equilibrium dialysis at pH 7.2.

The values for Deca represent an average of ^{14}C-labeled preparations (see text).

Results:

Equilibrium Dialysis: Equilibrium dialysis data obtained with the cholinergic agonists AcCh and Deca, and the inhibitor d-TC are presented in Figs. 1 to 3. One type of binding site was detected in each case, even at drug concentrations as low as 10^{-8} M (not shown); minor binding sites of higher affinity amounted to less than .5% of the total. AcCh displays the highest affinity, followed closely by d-TC, while Deca binds least strongly. The number of binding sites for AcCh, d-TC and Deca is approximately equal to about half the number of αBgt binding sites. Treatment of AcChR with αBgt prior to dialysis completely abolished binding of small ligands. The noncooperative nature of AcCh binding is shown in Fig. 4. Hill coefficients were also determined for the other ligands and found to be very close to unity. Table I represents average binding data, based on at least four experiments for each ligand.

Toxin Binding Protection: The rate of αBgt binding to AcChR is slowed down

Table II. Drug Profile of Purified AcChR.

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

The relative potency of various drugs to interfere with αBgt binding to AcChR was measured as described in Methods. Average values from two to six experiments for each ligand are given.

by cholinergic drugs (11). The effect of some ligands, at various concentrations on receptor-toxin complex formation is shown in Fig. 5. Half-maximal inhibition of toxin binding clearly is achieved at various concentrations for different ligands. Under the experimental conditions used the amount of toxin-receptor complex formed in one minute is not a measure of the initial rate of toxin binding; the I_{50} value obtained therefore does not give the dissociation constant. Nevertheless this I_{50} value permits estimation of the relative affinity of different ligands to the αBgt binding site. This relative affinity appears to be in good agreement with K_D values determined by equilibrium dialysis (Fig. 6). A variety of drugs were investigated for their potency to interfere with αBgt binding. Results are listed in Table II.

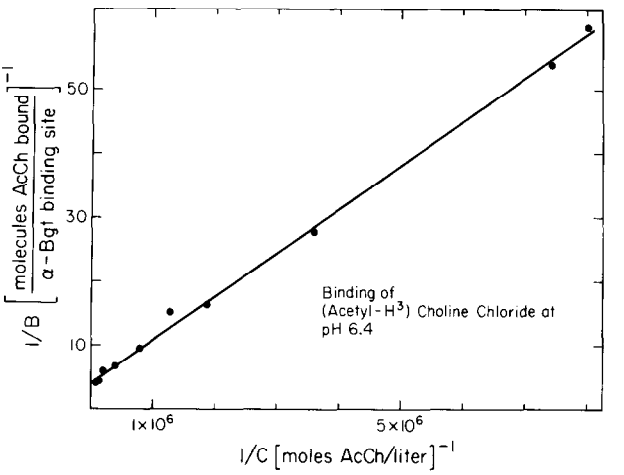

Figure 1: Double reciprocal plot of AcCh binding to purified AcChR at pH 6.4. Equilibrium dialysis was carried out as described in Methods. Ligand concentrations from 0.01 to 1.0×10^{-5} M are shown. The ordinate is expressed as molecules of ligand bound per Bgt site. See Discussion for pH effects.

The nicotinic agents d-TC, flaxedil and nicotine appear as much more powerful inhibitors than the muscarinic drugs atropine and pilocarpine, or the antiesterase eserine. It is noteworthy that at sufficient ligand concentrations toxin binding is completely inhibited.

Discussion:

The cholinergic nature of the innervation of the electric organ of Torpedo marmorata was first demonstrated by Feldberg et al. (12); this was corroborated by Lester (13) and Miledi et al. (14) who showed that electroplaque preparations were sensitive to αBgt and d-TC. O'Brien and his collaborators have thoroughly investigated the binding properties of particulate preparations of Torpedo marmorata and established that its AcChR is of the nicotinic type (1, 15).

In agreement with this, our data shown in Table II clearly indicate the nicotinic nature of the purified AcChR from Torpedo californica electroplax. This conclusion is also in agreement with the recent study of

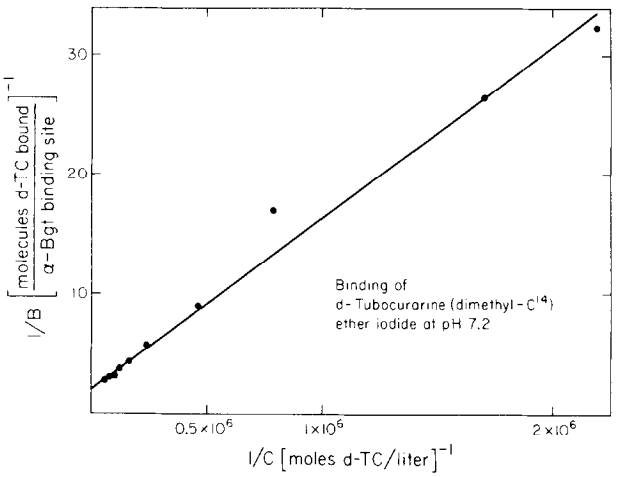

Figure 2: Double reciprocal plot of d-TC binding to purified AcChR at pH 7.2. Equilibrium dialysis was carried out as described in Methods. Ligand concentrations from 0.02 to 1.0 x 10^{-5} M are shown.

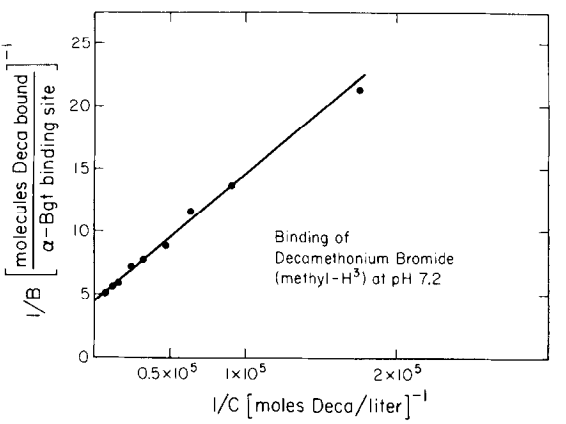

Figure 3: Double reciprocal plot of ^{3}H-Deca to purified AcChR. Equilibrium dialysis was carried out as described in Methods. Ligand concentrations from 5 to 250 x 10^{-6} M are shown. For a comparison of ^{3}H- and ^{14}C-decamethonium, see Discussion.

Eldefrawi et al. on acetylcholine binding to Lubrol WX-extracts from Torpedo marmorata electroplax (3). However, their findings contrast with ours in other respects: while they observe two types of AcCh binding with dissociation

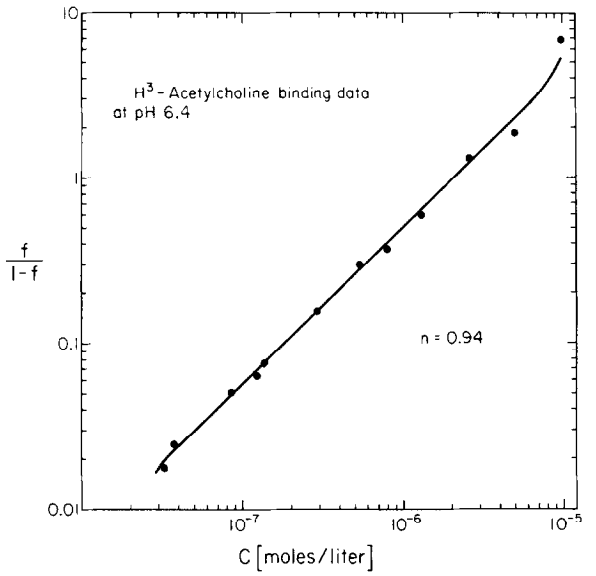

Figure 4: Hill plot of AcCh binding to purified AcChR. Data from the binding experiment described in Fig. 1 are presented over a concentration range of 0.003 to 1.0×10^{-5} M.

constants of 1.4×10^{-9} and 2.2×10^{-7} M, our data indicate a single site to which AcCh binds with a K_D of 2.3×10^{-6} M. They also report negative cooperativity and autoinhibition of AcCh binding at concentrations above 10^{-6} M; both of these observations are at variance with our findings.

Franklin and Potter (2) have studied the affinities of several small ligands to Triton-solubilized Torpedo marmorata membranes. Using a neurotoxin binding protection assay they were able to estimate dissociation constants; their value of 5×10^{-6} M for the K_I of d-TC is very close to the K_D of d-TC found in this study (6.4×10^{-6} M). Similarly K_I's for other ligands (carbamyl choline: 5×10^{-5} M; hexamethonium, 10^{-4} M; atropine, 5×10^{-4} M) agree well with our results for purified receptor.

It would appear unlikely that species differences or the presence of inactive membrane proteins can account for the discrepancy between our AcCh binding data and those of Eldefrawi et al. It is conceivable that a high-affinity binding site for AcCh may have been eliminated in our purification

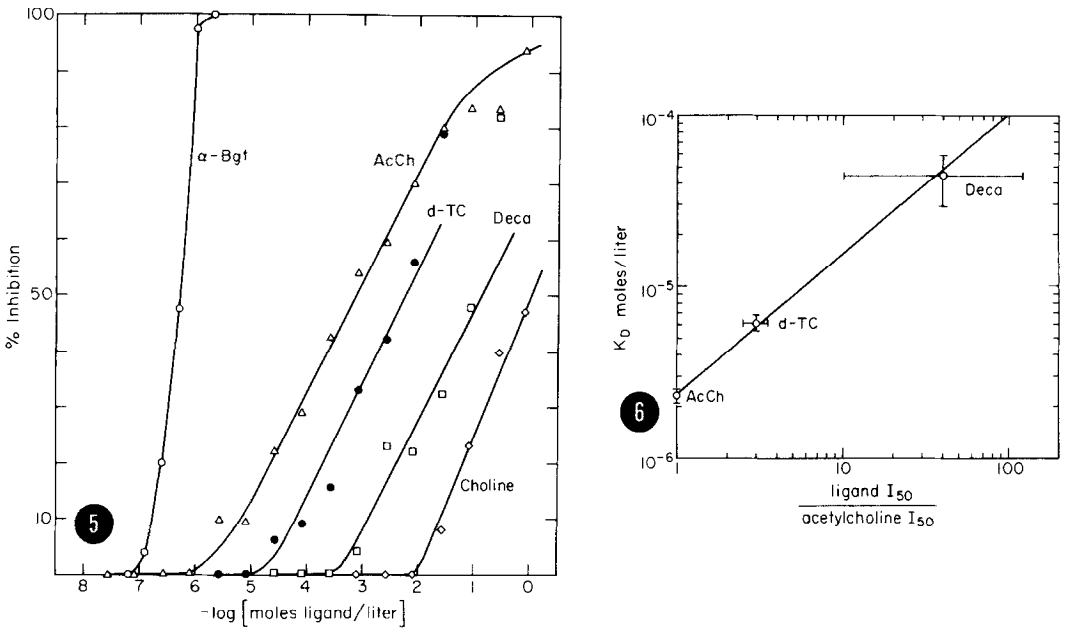

Figure 5: Effect of ligands on binding of αBgt to purified AcChR. The protection of AcChR against binding of ^{125}I-αBgt by native αBgt, AcCh, d-TC, Deca and choline at the indicated concentrations was studied as described in the text.

Figure 6: Comparison of data obtained by equilibrium dialysis and toxin binding protection studies.

procedure, but on the other hand, it remains to be seen if and how the organophosphates which O'Brien and associates use in 100 to 50,000 fold excess over AcCh affect the outcome of equilibrium dialysis experiments.

Meunier and Changeux have recently investigated the binding of decamethonium to purified AcChR from Electrophorus electricus (17). Their dissociation constant of 2.1×10^{-8} M is three orders of magnitude lower than that for Torpedo AcChR reported here, and confirms our earlier observation that small ligands inhibit αBgt binding to electric eel electroplax extracts at much lower concentrations than they do to solubilized Torpedo receptors (11). There is little disagreement concerning the total number of cholinergic binding sites extractable with nonionic detergents: 0.5-1.0 nmoles per gram of Torpedo

electric organ (14,3,15). The number of sites on the individual receptor molecule, however, remains unknown. It is apparent from Table I that about two molecules of αBgt bind per molecule of small ligand at pH 7.2. Lee (18) has shown that αBgt is a monomer in dilute solution with a molecular weight of 8,000; hence the observed ratio of bound αBgt to small ligands probably reflects the pH (7.2) and ionic strength (200 mM) utilized, as preliminary studies (T. Moody and M.A. Raftery, unpublished) indicate that, at higher pH and/or lower ionic strength, the ratio of toxin to small ligand binding sites approaches unity. Changeux and his collaborators have reported a ratio of 0.63 Deca molecules bound per toxin binding site for the AcChR of the electric eel (17). This value was obtained by correcting for a biologically inactive component in the tritiated Deca preparation. We studied the binding of ^{14}C-Deca as well as ^{3}H-Deca and found that there was an increase in the apparent occupation of toxin binding sites from 30 to 55%, in agreement with the observation of Meunier and Changeux. The value presented in Table I is an average of ^{14}C-Deca experiments.

Published experiments (19,20) on binding of small ligands to membrane preparations of Torpedo marmorata and Electrophorus electricus have not been interpreted as indicating cooperative binding; similarly decamethonium binding to Torpedo californica electroplax membranes appears to be noncooperative (T. Moody and M.A. Raftery, unpublished). Meunier and Changeux have recently reported that binding of Deca to purified eel AcChR follows the classic Langmuir isotherm (17), which agrees with the Hill coefficient of unity that we observe for purified Torpedo californica AcChR. Thus receptor solubilization and purification probably does not affect the noncooperativity of binding observed with intact membranes. These are significant findings in view of the fact that the cooperative response of excitable membranes to drugs is well documented (for a review, see Ref. 21). Since it has been shown that the conductance of the postsynaptic membrane changes in a cooperative fashion with drug concentration (21,22), cooperative

effects must occur somewhere between application of the cholinergic agonist and the opening of ion channels.

Two types of models have been proposed which locate cooperativity at different levels of organization. Karlin (23) and Edelstein (24) have treated the receptor as an allosteric protein, suggesting that interactions between subunits within the receptor molecule may give rise to cooperativity. Changeux et al. (25) have considered cooperativity as due to interactions between repeating lipoprotein units in the membrane. Since the solubilized Torpedo AcChR, though still composed of polypeptide subunits (8), does not exhibit cooperative ligand binding, AcChR cannot be viewed as an allosteric protein in terms of cholinergic ligand binding and the reason for the sigmoidal concentration-effect curves observed in vivo must be sought elsewhere.

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