Studies on Nicotinic Acetylcholine Receptors in Mammalian Brain

INTERACTION OF SOLUBILIZED PROTEIN WITH CHOLINERGIC LIGANDS*

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Binding of α-bungarotoxin, labeled with $^{125}$I, has been studied in detergent extracts and affinity purified acetylcholine receptor from rat cerebral cortex. Binding to detergent extracts is saturable and appears to be due to one class of binding sites present at a level of 0.27 pmol/mg of protein. The association constant is $2 \times 10^7$ liters mol$^{-1}$. Competition with cholinergic ligands indicates that toxin binding to both detergent solubilized and affinity purified receptor retains its nicotinic nature. Values for the ligand concentrations required to produce 50% inhibition of extent and rate of toxin binding are presented.

In recent years, rapid progress has been made in elucidating the nature of the nicotinic acetylcholine receptor† from a wide range of sources (see Ref. 1 for a collection of pertinent papers). This advance has been made possible by the introduction by Changeux (2) of the use of α-neurotoxins isolated from the venom of various elapid snakes. These neurotoxins bind specifically and with high affinity to the nAChR (3, 4). They, especially α-bungarotoxin, obtained from Bungarus multicinctus, the Formosan krait, and cobra toxin from various Naja species, have been used extensively to identify and quantitate the nAChR (5–8).

The techniques for the assay, solubilization, and purification of the nAChR were developed for the electric organ of electric eels and other fish, a tissue that is highly enriched in the nAChR. These techniques were then successfully adapted to study the nAChR of the neuromuscular junction in the intact tissue and after its removal and culture (1).

Study of the nAChR in cerebral cortex has proven to be much more difficult because the predominant form of the AChR is muscarinic and not nicotinic (9). Farrow and O'Brien (10) found a high affinity binding site for atropine, a muscarinic ligand, at a concentration of 89 pmol/g of tissue in rat cerebral cortex; Yamamura and Snyder (11), using 3-quinuclidinyl benzylate, a potent central muscarinic antagonist, found a density of muscarinic sites of 65 pmol/g of rat brain. In 1972, Moore and Loy (12) demonstrated the presence of an α-Btx binding component in hog brain. The following year Salvaterra and Moore (13) determined the number of α-Btx binding sites as 3.4 pmol/g of rat cerebral cortex. These values were confirmed and extended to a number of brain regions and subcellular fractions in a study by Salvaterra et al. (14). These levels are very low as compared to the muscarinic AChR in the same preparation, to the 1 to 2 nmol/g of tissue in Electrophorus electricus (8), or to 0.5 to 1 nmol/g of tissue in Torpedo californica (5).

Eterovic and Bennett (15) reported on the binding of tritiated α-Btx to crude mitochondrial fractions from rat cerebral cortex and the inhibition of binding by a variety of cholinergic drugs. Moore and Brady (16) and McQuarrie et al. (17) studied the binding of α-Btx, labeled with $^{125}$I, to crude membrane preparations of rat cerebral cortex. The latter study, as well as a recent one by Schmidt (18), reported on the inhibition of α-Btx binding to such preparations by a number of cholinergic and noncholinergic drugs.

Salvaterra and Mahler (19), Lowy et al. (20), and Moore and Brady (16) have described the solubilization of the nAChR from rat cerebral cortex by detergents, i.e. 1% Triton X-100 and 1% Emulphogene; however, these studies do not deal with the inhibition of toxin binding by cholinergic drugs.

This paper reports on some $^{125}$I-α-Btx binding properties of detergent extracts of a crude membrane preparation from rat cerebral cortex. We also report on the inhibition of rate of $^{125}$I-α-Btx binding by some cholinergic drugs to the solubilized AChR purified according to Salvaterra and Mahler (19).

EXPERIMENTAL PROCEDURES

Preparation of Membrane-bound Receptors—The insoluble material containing the membrane-bound receptor was prepared from male Sprague-Dawley rats (30 to 35 days old) (Harlan Industries, Cumberland, IN) according to McQuarrie et al. (17).

Solubilization and Purification—The membrane-bound receptor...
was solubilized by 1% Triton X-100 containing 50 mM NaCl, 50 mM Tris, pH 7.4, containing 10^{-3} M phenylmethylsulfonyl fluoride as described by Salvaterra and Mahler (19). Some experiments were performed on crude membrane preparations by first adding the insoluble material with 10 times its volume in 20 mM sodium phosphate, pH 7.4, 1% Triton for 4 h. The suspension was centrifuged at 125,000 \times g for 1 h. The supernatant was removed by aspiration and represented the Triton extract.

The n-toxin was purified by affinity chromatography using a Naja naja siamensis a-neurotoxin as the ligand as described by Salvaterra and Mahler (19).

Preparation and Iodination of Toxin — α-Bungarotoxin was purified from the venom of Bungarus multicinctus by the method of Mebs et al. (21). The purified protein showed a single homogeneous band on electrophoresis (Beckman Microzone).

The purified toxin was iodinated using either chloramine-T (22) (as described in Ref. 14) or the solid state lactoperoxidase method (23) (as described in Ref. 19). The concentration of the labeled toxin was determined by the fluororescamine method of Bohlen et al. (24).

The specific activity was generally about 3 \times 10^8 Ci/mol.

Preparation of Affinity Resin — The a-neurotoxin was isolated from the venom of Naja naja siamensis according to Mebs et al. (21). The a-toxin was coupled to CNBr-activated Sepharose 4B according to the directions of the manufacturer (Pharmacia).

DEAE-Cellulose Filter Binding Assay — Binding of labeled α-Btx to Triton X-100 solubilized and purified AChR was assayed by the DEAE-cellulose filter disc assay of Schmidt and Raftery (25). In equilibrium studies, the sample was incubated 1 h with the labeled toxin. In rate studies, aliquots of 50 or 100 μl were taken at times 2.5, 5, 7.5, and 10 min. In inhibition experiments, the sample was incubated with the drug at the appropriate concentration for 30 min prior to addition of labeled toxin.

Other — Protein was determined by the method of Lowry et al. (26) using bovine serum albumin as standard. Radioactivity was determined with a Beckman Biogamma scintillation counter at an efficiency of 75%.

Materials — B. multicinctus and Naja naja siamensis venom were purchased as lyophilized powders from the Miami Serpentarium. I^{131} was purchased as carrier-free Na^{131}I in 0.1 N NaOH from New England Nuclear. All other chemicals and reagents were of reagent grade or better and purchased from commercial sources.

RESULTS

A binding isotherm for I^{125}I-α-Btx to the Triton X-100 extract containing the AChR receptor in solubilized form is presented in Fig. 1 as picomoles of toxin bound per mg of protein versus total toxin added. The data cover toxin concentrations from 4.5 to 450 nM. It is apparent that under these conditions a set of sites becomes saturated at an α-Btx concentration of approximately 100 nM. A Scatchard plot (27) (not shown) reveals the presence of a single set of binding sites. The concentration of AChR as determined from the saturation of toxin binding is 0.27 pmol/mg of protein which corresponds to approximately 80% of the total AChRs present in the particulate fraction of crude membrane preparations. The apparent association constant $K_a$, as calculated from the concentration at which 50% saturation occurs, is found to be 2 \times 10^3 M^{-1}.

In Fig. 2 are presented the extent of inhibition at equilibrium of α-Btx binding in Triton extracts by several acetylcholine agonists and antagonists. The nicotinic antagonist, $d$-tubocurarine, the acetylcholine agonist, carbamylcholine, and acetylcholine itself are all more effective inhibitors of $I^{125}I$-α-Btx binding than are the muscarinic ligands, atropine and oxotremorine.

In Fig. 3 we present the inhibition of the rate of binding of $I^{125}I$-α-Btx in similar Triton X-100 extracts by a series of cholinergic drugs. The most effective inhibitor is $d$-tubocurarine, followed closely by carbamylcholine and acetylcholine. The nicotinic antagonist, gallamine (flaxedil), and the muscarinic antagonist, atropine, are much less effective, while hexamethonium and the muscarinic agonist, scopolamine, are essentially without effect. Unlabeled α-Btx inhibits the rate of binding of 50% at a concentration somewhat less than 2.4 \times 10^{-9} M.

The data of Fig. 4 represent the inhibition by these drugs of the rate of this reaction with the affinity purified AChR. The most effective inhibitor is acetylcholine; much less effective

![Fig. 1. Binding isotherm for α-Btx labeled with I^{125}I to a Triton X-100 extract from rat cerebral cortex. 0.3-ml aliquots of Triton extracts (4 mg/ml) from two different preparations were incubated with the indicated toxin concentrations and assayed for binding as described under "Experimental Procedures."](http://www.jbc.org/)
Fig. 4. Inhibition of the rate of binding of radioiodinated α-neurotoxin to affinity purified AChR by various cholinergic ligands. 0.4-ml samples were preincubated for 30 min with the appropriate ligand. Rate of binding was determined as described under "Experimental Procedures." Samples were preincubated with 5 × 10^{-4} M eserine before adding acetylcholine. •, carbamylcholine; ○, atropine; △, scopolamine; ▲, gallamine; ◦, hexamethonium; ▼, decamethonium; □, acetylcholine.

Table I

| Solubilized | Affinity-purified |
|-------------|-------------------|
| Equilibrium | Rate              | Equilibrium | Rate |
| d-Tubocurarine | 1 × 10^{-4} | 3 × 10^{-5} | 2 × 10^{-5} | <10^{-6} |
| Acetylcholine   | 1 × 10^{-3} | 2 × 10^{-6} | <10^{-3} | 6.3 × 10^{-4} |
| Carbamylcholine | 3 × 10^{-4} | 8 × 10^{-4} | 6 × 10^{-5} | 1.3 × 10^{-4} |
| Gallamine      | 2 × 10^{-4} | 2 × 10^{-4} | 1.6 × 10^{-4} |
| Hexamethonium  | >10^{-3} | 4.0 × 10^{-4} |
| Atropine       | 2 × 10^{-5} | 3 × 10^{-5} | 1 × 10^{-5} | >10^{-3} |
| Scopolamine    | >10^{-3} | >10^{-3} |
| Oxotremorine   | 2 × 10^{-4} |

* Data taken from Ref. 19.

are carbamylcholine, decamethonium, and gallamine, while the least effective are hexamethonium, atropine, and scopolamine. Unlabeled α-Btx inhibits the rate of binding by 50% at a concentration of 2.4 × 10^{-4} M.

The concentrations of the various ligands required to produce 50% inhibition are summarized in Table I.

**DISCUSSION**

We have previously shown that crude membrane preparations from rat cerebral cortex contain two classes of bound α-Btx binding sites of high and low affinity (17). Weber et al. (28) and Hess et al. (29) have also reported evidence that the detergent solubilization of the membrane-bound AChR exposes additional sulphydryl groups which are rapidly oxidized in Triton X-100.

Table II presents data, taken from the literature, on the corresponding values for the membrane-bound AChR in rat cerebral cortex. This finding may provide an explanation for the apparent interconversions of the two classes of receptors just discussed.

Using the rates of association and dissociation between α-Btx and solubilized AChR for the calculation of binding constants, Moore and Brady (16) and Lowy et al. (20) also find only one class of α-Btx binding sites in detergent extracts of rat cerebral cortex. Although the association constant determined from equilibrium studies as in the present investigation is an underestimate of the true value, this value can still be used for comparisons with similar measurements on the membrane-bound receptor.

Very recently, Maelicke et al. (34) reported the presence of two classes of sites in affinity purified AChR prepared from E. electricus as revealed by the rate of association and dissociation of Naja naja siamensis α-neurotoxin. Both classes exhibit identical rates of association but different rates of dissociation, the more rapidly dissociating component becoming only apparent with increasing occupancy of sites. The technical advances in the DEAE-filter disc assay developed in that study made possible the study of toxin-receptor interactions over a wide range of concentrations, especially at low toxin concentrations. The low concentration of AChR present in cerebral cortex made studies at low α-Btx concentrations not feasible, thereby perhaps preventing the detection of the high affinity site. The binding of α-Btx is essentially irreversible while that of the Naja naja siamensis α-neurotoxin is reversible (3, 4) which would make the detection of different rates of dissociation of α-Btx receptor complexes much more difficult than that of the Naja toxin-receptor complexes.

Table I summarizes the concentration of the cholinergic ligands required to produce 50% inhibition of radioiodinated α-Btx binding at close to equilibrium and of the rate of its binding to solubilized and affinity purified AChR of rat cerebral cortex. In Table II are presented data, taken from the literature, on the corresponding values for the membrane-bound AChR in the same tissue. It is apparent that the AChR fully retains its nicotinic character during solubilization and subsequent purification by affinity chromatography. Muscarinic ligands, such as the agonist, oxotremorine, and the antagonists, atropine and scopolamine, are much less effective in reducing α-Btx binding in all cases than is acetylcholine itself or the classic nicotinic ligands, tubocurarine and carbamylcholine. Hexa-
enhanced by a factor of 10 under these conditions. Upon activation of AChR, while the effectiveness of carbamylcholine is greater than that of nicotine, the nicotinic acetylcholine antagonist, to inhibit α-Btx binding is limited. The ability of gallamine, a nicotinic antagonist, to inhibit α-Btx binding is much lower when determined by measuring their effect on muscle end plates (35). Very recently, Moore and Brady (36) reported on the solubilization of the AChR from rat cerebral cortex by the detergent Emulphogene BC 720 and on some of the binding characteristics of these detergent extracts. The inhibition of the extent of 125I-α-Btx binding by d-tubocurarine and hexamethonium was very similar to that reported by Dolly and Barnard (37) found a slight, uniform increase in affinity for some of the binding characteristics of these detergent extracts. This phenomenon. The concentration at which 50% inhibition occurs with tubocurarine, acetylcholine, and carbamylcholine is much lower when determined by measuring their effect on rate rather than the extent of 125I-α-Btx binding to the solubilized receptor. After its purification similar effects are seen for tubocurarine and acetylcholine, while carbamylcholine appears somewhat less effective in reducing the rate of toxin binding than it is under equilibrium conditions. Similar results were found by Clunie and co-workers (26, 39) on the AChR from E. electricus and by Raftery and co-workers (25) reported little change in the effectiveness of the various cholinergic drugs upon solubilization of the AChR from the electric organ of E. electricus.

When studying competitive inhibition of binding of an essentially irreversible ligand such as α-Btx, the inhibition of the rate of its association by the competitive ligands provides a much more sensitive measure of their relative affinities than do measurements of displacement close to equilibrium (5). The data presented in Table I clearly demonstrate this phenomenon. The concentration at which 50% inhibition occurs with tubocurarine, acetylcholine, and carbamylcholine is much lower when determined by measuring their effect on rate rather than the extent of 125I-α-Btx binding to the solubilized receptor. After its purification similar effects are seen for tubocurarine and acetylcholine, while carbamylcholine appears somewhat less effective in reducing the rate of toxin binding than it is under equilibrium conditions. Similar results were found by Clunie and co-workers (26, 39) on the AChR from E. electricus and by Raftery and co-workers (25) on the AChR from Torpedo californica.

After extensive purification by affinity chromatography the solubilized AChR retains its affinities for all cholinergic ligands with the exception of carbamylcholine, the affinity of which reverts to a value resembling that found with the membrane-bound AChR. Dolly and Barnard (37) reported no change between solubilized and affinity purified AChR from muscle, although they raised the α-Btx concentration 10-fold to determine the rate of binding to the purified receptor. Comparable studies on the AChR from the electric organ have not been reported in the literature. In Table III are presented data taken from the literature for the values of the concentration of cholinergic ligands required for 50% inhibition of the rate of binding of labeled α-neurotoxin to purified AChR. From a comparison of these values with the corresponding ones summarized in Table I, we conclude that the AChR found in cerebral cortex is similar but not identical with that found in the electric organ of electric fish.

Maelicke et al. (34) have recently also completed an exhaustive analysis of the binding of Naja naja siamensis α-neurotoxin to AChR from the electric organ of E. electricus after its purification by affinity chromatography and the inhibition of that binding by various cholinergic ligands. The use of an α-neurotoxin with a reversible, instead of an essentially irreversible, binding mode, such as α-Btx, enabled the authors to develop relationships between the amount of labeled toxin bound to the AChR, and Ki, the inhibitor dissociation constant of the cholinergic ligand, determined under equilibrium conditions. These relationships were derived from basic thermodynamic arguments with a minimum of assumptions; Ki values calculated from these expressions represent the most accurate determinations of these values since they are based on firmer theoretical grounds than assuming Ki to be equal to the concentration at which 50% inhibition occurs. The values reported by Maelicke et al. (34) were generally at least 10 times lower than previously published values, obtained by a variety of techniques. Although it is clear that this method for

### Table II

|                | Crude mitochondrial fraction (36) | Crude mitochondrial fraction (14) | Hemogoneate (18) | PBR (17) | P2 (17) |
|----------------|----------------------------------|----------------------------------|------------------|-----------|---------|
| d-Tubocurarine | 10^-4                            | 5 × 10^-7                        | 1.9 × 10^-6      | 10^-5     | 2 × 10^-5 |
| Acetylcholine  |                                  |                                  | 3 × 10^-3        | 10^-6     | 10^-6   |
| Carbamylcholine| 10^-4                            |                                  | 9 × 10^-6        | 10^-6     | 10^-6   |
| Gallamine      | 3.5 × 10^-6                      |                                  | 9 × 10^-6        | 10^-6     | 10^-6   |
| Hexamethonium  | 6.2 × 10^-5                      |                                  | 2 × 10^-3        | 10^-3     | 9 × 10^-4 |
| Atropine       | 10^-2                            | 8 × 10^-4                        | 1.6 × 10^-3      | 10^-3     | 9 × 10^-4 |
| Oxotremorine   | 2.0 × 10^-3                      |                                  | 2.9 × 10^-3      | 10^-3     | 9 × 10^-4 |

### Table III

| Source                  | Muscle (37) | Electroplax (41) | Torpedo coli. (42) | Torpedo coli. (43) |
|-------------------------|-------------|------------------|--------------------|--------------------|
| d-Tubocurarine          | 3.3 × 10^-7 | 3.9 × 10^-7      | 5.6 × 10^-7        | 1.0 × 10^-7        |
| Acetylcholine           | 1 × 10^-5   | 7.7 × 10^-6      | 2.5 × 10^-6        | 2.5 × 10^-6        |
| Carbamylcholine         | 0.9 × 10^-5 | 1.9 × 10^-4      | 4.5 × 10^-3        | 4.5 × 10^-3        |
| Gallamine               | 1.3 × 10^-7 | 2 × 10^-7        | 2 × 10^-6          | 2 × 10^-6          |
| Hexamethonium           | 6.2 × 10^-5 | 3.3 × 10^-5      | 2 × 10^-6          | 2 × 10^-6          |
| Atropine                |             |                  |                    |                    |
the determination of inhibitor dissociation constants is the most accurate, it is not readily applicable to brain AChR due to the much lower concentration of AChR in this tissue as compared to that found in the electric organ. The preparation of sufficient purified AChR to carry out the evaluation of Ki values according to that method would represent an enormous effort in terms of time and cost. The data presented in this report are more or less self-consistent and do allow one to detect changes in binding properties during the various stages of purification. Since they were determined under conditions similar to many of those previously reported for AChR derived from a variety of tissues, they should still prove useful for comparison between the AChR in brain and other vertebrate tissues.

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