AFFINITY OF CHOLINERGIC LIGANDS FOR THE PARTIALLY PURIFIED ACETYLCHOLINE RECEPTOR FROM MAMMALIAN SKELETAL MUSCLE

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1. Introduction

[^{3}H]Acetylated-α-bungarotoxin has been used to determine the numbers, densities and precise location of acetylcholine receptor (ACh·R) molecules in normal and denervated mammalian muscle [1–4]. Two labeled components specifically binding α-bungarotoxin (BuTX) have been solubilized from rat diaphragm muscle and shown to have apparent molecular weights (by gel filtration in 0.2% Triton X-100) of 550 000 and 200 000, respectively [4]. Only the former is found at the endplates and is thought to contain the native ACh·R. After denervation, a 20-fold increase occurs in a BuTX-binding component with an apparent mol. wt. of 550 000, but this is then distributed throughout the whole muscle. Unlabeled ACh·R with the same mol. wt. has been solubilized from denervated mammalian muscle, extensively purified (100–200 fold) and shown to bind cholinergic ligands [4–6]. A detailed description of the purification and properties of the ACh·R from denervated cat leg muscles will be described elsewhere [7,8]. A partially purified preparation of ACh·R from rat denervated diaphragm has been reconstituted into artificial lipid membranes [9]. Here we show, by the protective effects on the binding of BuTX, that cholinergic agonists and antagonists bind with high affinities to this partially purified ACh·R preparation from cat denervated muscles.

2. Materials and methods

2.1. *Preparation of [^{3}H] triacetylated-α-BuTX*

Pure BuTX was purified and acetylated with [^{3}H]acetic anhydride, as described previously [1]. By further purification steps a homogeneous species, [^{3}H]triacetylated-α-BuTX ([^{3}H]BuTX) of specific activity 4.5 Ci/mmol, was obtained, which displayed the same specificity but somewhat lower affinity than the native toxin [10].

2.2. *Assay of [^{3}H]BuTX-binding activity*

Toxin-binding activity was measured at 25°C using 16 nM [^{3}H]BuTX in 0.2% Triton X-100/10–25 mM KH_2PO_4–K_2HPO_4, pH 8, by a method utilizing DEAE-cellulose filter discs [8,11,12]. For experiments with acetylcholine or carbamylcholine, the receptor preparation used was preincubated with 1.5×10^{-5} M DFP (0.3×10^{-5} M final conc. in control and test reaction mixtures) at 5°C for at least 30 min before addition of ligand. The buffered-receptor solutions were always incubated for at least 15 min at 5°C with the ligands before the reaction was started by adding [^{3}H]BuTX. After thorough washing, the filters were dried and counted by liquid scintillation at 34% efficiency, in a toluene-based medium containing 10% (v/v) Soluene-100.

2.3. *Preparation of the ACh·R*

Cat lower-leg muscles were denervated by scission of the sciatic nerve under anesthesia, and dissected out 4 weeks later. The ACh·R was extracted using 1.5% Triton X-100/50 mM KH_2PO_4–K_2HPO_4, pH 8, gel-filtered on Sepharose 6B and concentrated by ultrafiltration [4]. The preparation could be stored at −15°C for 4 days without appreciable loss in toxin-binding activity. For some ligand-binding experiments, an ACh·R preparation purified 110-fold by affinity

chromatography was used [6–8]. Preparations of the ACh-R purified by gel filtration and affinity chromatography bound 8 and 150 nmoles of [^{3}H]BuTX per gram protein, respectively.

2.4. *Chemical sources*

Acetylcholine iodide (ACh), carbamylcholine chloride, d-tubocurarine chloride (d-TC), choline chloride, hexamethonium bromide, atropine sulfate and DFP: Sigma; decamethonium bromide and pilocarpine hydrochloride: K and K Labs.; Triton X-100 and Soluene-100: Packard.

3. Results

By means of the DEAE-cellulose filter assay method and [^{3}H]BuTX of high specific activity, it was possible to measure accurately the effects of various ligands on the rate of [^{3}H]BuTX binding to the ACh-R, in preparations containing relatively small amounts of receptor. 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 range of concentrations [8].

Fig. 1 shows the binding of [^{3}H]BuTX to ACh-R as a function of the concentration of radioactive toxin

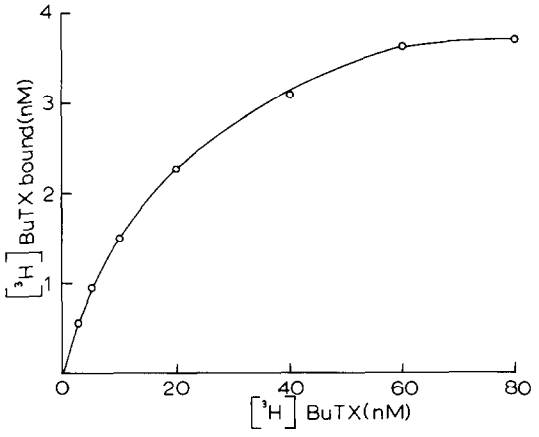

Fig. 1. Binding of [^{3}H]BuTX to partially purified ACh-R as a function of toxin concentration. After 20 min of incubation at 25°C, the bound [^{3}H]BuTX was determined using the DEAE-cellulose disc assay. Blank values, determined for each concentration of toxin, have been subtracted.

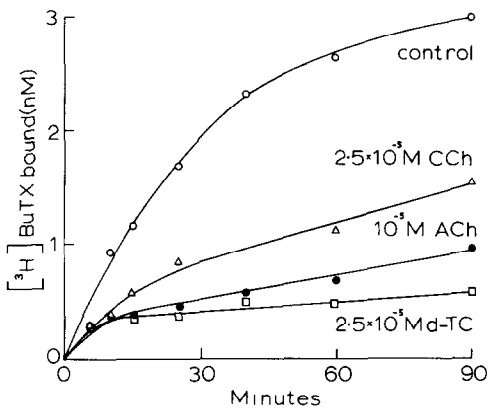

Fig. 2. Binding of [^{3}H]BuTX (16 nM) to partially purified ACh-R (3 nM) at 25° alone (○), and in the presence of carbamyl-choline (△), ACh (●) and d-TC (□).

used. The results show that only a single saturable site for [^{3}H]BuTX was detectable. The reaction of [^{3}H]BuTX with ACh-R, alone and in the presence of carbamylcholine, ACh or d-TC, is illustrated in fig. 2. The ACh-R preparation showed high affinity for the ligands but the kinetics were complicated by the fact that a fraction of the activity (approx. 12% of total), which displayed the usual fast reaction rate with [^{3}H]BuTX, could not be protected by cholinergic ligands (ACh, carbamylcholine, decamethonium or d-TC), even at high concentrations (e.g., 2.5 X 10^{-5} M d-TC; fig. 2). Various types of control (e.g., heat-inactivated receptor, omission of the receptor, or reaction performed at 5°C for 1 min) tested did not yield this anomalous contribution. Hence, the ligand-insensitive BuTX-binding component is inactive in the conditions where the specific (ligand-sensitive) component is itself inactive and is not an artefact of the assay system. Since this fraction was independent of all ligands tested, it was taken to be a minor second component and its contribution was subtracted in every case. When this was done (fig. 3) the reaction is essentially linear for the first 25 min at 3–4 nM receptor concentration, so that initial rates could be measured with accuracy. Similar competition experiments were performed with ACh-R, purified by gel filtration and affinity chromatography, for a total of 5 ligands, and the protection constants were estimated (table 1) using the equation [13]:

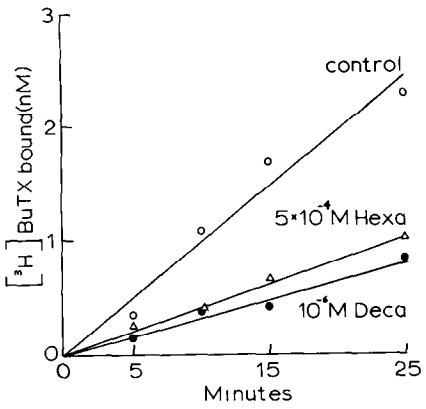

Fig. 3. Rates of [^{3}H]BuTX binding to ACh·R alone (○) and in the presence of hexamethonium (△) and decamethonium (●).

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

where v and v_i are the initial rates measured in the absence and presence of ligand, respectively; L is the concentration of ligand, and K_p is the protection constant of the ligand. In using this equation we assume that the toxin can bind only to the free ACh·R; v_i is then proportional to the concentration of sites which are not occupied by the ligand, and K_p is equal to the dissociation constant of the ligand–ACh·R complex. That this equality holds, has been shown in the case of electroplaque ACh·R reacting with a cobratoxin, by direct binding measurements with several ligands [14].

The muscarinic ligands, atropine and pilocarpine, when used at concentrations (1×10^{-5} and 5×10^{-6} M, respectively) that normally block muscarinic-type receptors, showed no protection. Likewise, choline (2×10^{-5} M) did not affect the rate of [^{3}H]BuTX binding to ACh·R.

4. Discussion

The present results show that detergent-solubilized ACh·R, partially purified from cat denervated leg muscles, binds cholinergic agonists and antagonists with high affinities. Similar binding constants were obtained (in the cases of ACh, carbamylcholine and d-TC) for the ACh·R which had been purified by affinity chromatography. The apparent binding

Table 1
Apparent protection constants for ACh·R Protein purified from cat denervated leg muscles

	Gel-filtered ACh·R	ACh·R purified by affinity chromatography	Electrophorus membrane ACh·R**
	(M)	(M)	(M)
Agonists			
Acetylcholine	1.7×10^{-6}*	1×10^{-6}*	1.5×10^{-6}
Carbamylcholine	1.0×10^{-5}*	0.9×10^{-5}*	4.0×10^{-5}
Decamethonium	0.5×10^{-6}		0.8×10^{-6}
Antagonists			
d-Tubocurarine	1.5×10^{-7}	3.3×10^{-7}	1.7×10^{-7}
Hexamethonium	3.4×10^{-4}		0.6×10^{-4}

Apparent protection constants (K_p) were estimated (see text) by following the effect of the ligand on the initial rate of [^{3}H]BuTX binding to partially purified ACh-R at 25°C.

* In the presence of 0.3×10^{-5} M DFP.

** Values given for comparison: taken from Weber and Changeux [14], measured by protection against the binding of [^{3}H]α-neurotoxin (*Naja nigricollis*), at 22°C in Ringer's solution.

constants obtained for the antagonists are very similar to those obtained for both membrane-bound and purified ACh·R of the *Electrophorus* electroplaque [14,15]. However, the affinities obtained for the agonists are similar only to those for membrane-bound *Electrophorus* ACh-R, since solubilization of the ACh-R from membrane fragments of *Electrophorus* causes an increase of 10–50 fold in its affinity for agonists [16]. Different affinities for these ligands, and their behavior upon solubilization have been reported by several laboratories for the ACh·R of *Torpedo* electroplax [14,17–19]. We, therefore, presume that the denervated muscle receptor is similar in binding properties to that of *Electrophorus*, but does not show the change to much higher affinity for ligands upon release from its state in the membrane.

It is interesting that only a small proportion of ($< 15\%$) of the [^{3}H]BuTX-binding activity of this solubilized muscle receptor is insensitive to cholinergic ligands. For the ACh·R of cultured myoblasts, a similar small resistant fraction is apparent in the data of Patrick et al. [20]. In contrast, in the intact innervated muscle, 50% of the toxin-binding sites at the endplates are readily protected by d-TC [1]. The d-TC-insensitive fraction is only 35% in the intact denervated mouse diaphragm muscle [21]. As 80% of the total BuTX-binding sites are solubilized by detergent [4] it is possible that the d-TC-insensitive fraction remains insoluble, or is a different component that is not purified with the true ACh-binding receptor. The affinity of the muscle receptor for d-TC found here is an agreement with that estimated pharmacologically by a null-point method: 3.9×10^{-7} M (at 30°C) for innervated hamster diaphragm [22]. Since the denervated muscles show in situ a lower apparent affinity for d-TC [21], possibly a change in the direction of increased affinity for this antagonist occurs upon solubilization.

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