BINDING PROPERTIES OF ACETYLCHOLINE RECEPTORS EXTRACTED FROM NORMAL AND FROM DENERVATED RAT DIAPHRAGM

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Received 23 September 1974

1. Introduction

When a muscle is denervated, acetylcholine sensitivity, normally restricted to the endplate region of the muscle fiber, spreads over the entire surface [1,2]. This change in acetylcholine sensitivity is not based on a redistribution or modification of existing receptor molecules, but on the appearance of new ones [3–5]. Denervation thus appears to be a simple procedure to induce the synthesis of acetylcholine receptors (AcChR) and to make them available in quantities sufficient for detailed biochemical and even protein-chemical investigations. The utilization of the denervation-induced receptor for the study of the nature and dynamics of the acetylcholine receptor ordinarily occurring in the postsynaptic membrane however is predicated on the identity of these two types of molecule. At present it is not known whether they are the same. In this communication we wish to report on experiments designed to compare the drug binding properties of detergent-solubilized receptors from normal as well as from denervated muscle. In contrast to earlier reports, drug affinities of the two types of receptor were found to be indistinguishable.

2. Materials and methods

2.1 *Preparation of muscle extracts*

Whole diaphragms were excised from control rats weighing approximately 120 g; denervated hemidiaphragms were obtained from rats of similar age, one, two, or four weeks after a unilateral phrenicotomy had been performed. No attempt was made to divide the muscle into endplate-free and endplate-containing segments. Receptors were solubilized with Triton X-100 from normal and denervated muscle following the protocol of Berg et al. [4]. Extracts from normal diaphragms were dialyzed against 10 mM sodium phosphate pH 7.4, 0.1% Triton X-100, 0.02% sodium azide; extracts from denervated muscle were diluted 50-fold into the same buffer.

2.2 *Toxin binding rate assay*

α-Bungarotoxin (α-Bgt) was purified from *Bungarus multicinctus* venom (*Miami Serpentarium*) and labeled with ^{125}I using the chloramine T procedure; unreacted toxin was then removed from the iodinated product by chromatography on CM-cellulose. The specific radioactivity of the iodotoxin preparations varied from 5 to 10×10^{17} cpm per mole, depending on batch and age; the biological activity was close to 100%, as measured by its ability to bind to an excess of purified AcChR from *Torpedo Californica*. Toxin binding to muscle extracts was measured by the DEAE-cellulose paper disk method [6]. When AcChR (2×10^{-11} M) was incubated with [^{125}I] α-Bgt (4×10^{-11} M) at room temperature, in 10 mM sodium phosphate pH 7.4, 0.1% Triton, 0.02% sodium azide, the binding reaction went to completion in about one hr. To approximate initial rate conditions the reaction was terminated before half saturation was reached; this took about 10 min. For binding studies, varying concentrations of several ligands were included in the incubation mixture.

3. Results and discussion

Fig. 1 shows the development of AcChR in denervated rat diaphragm. Two weeks after phrenicotomy

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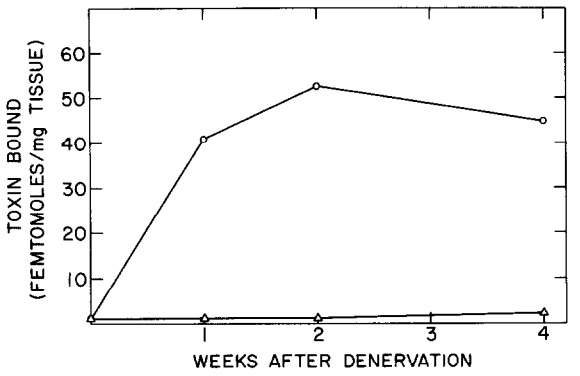

Fig.1. Effect of denervation on receptor concentration in rat diaphragm. Unilateral denervation was performed by excising a section of the left phrenic nerve. At the indicated times after phrenicotomy the entire diaphragm was removed, divided into denervated (o—o—o) and control (△—△—△) halves, extracted, and assayed for α-Bgt binding. Zero time values were obtained from a normal diaphragm.

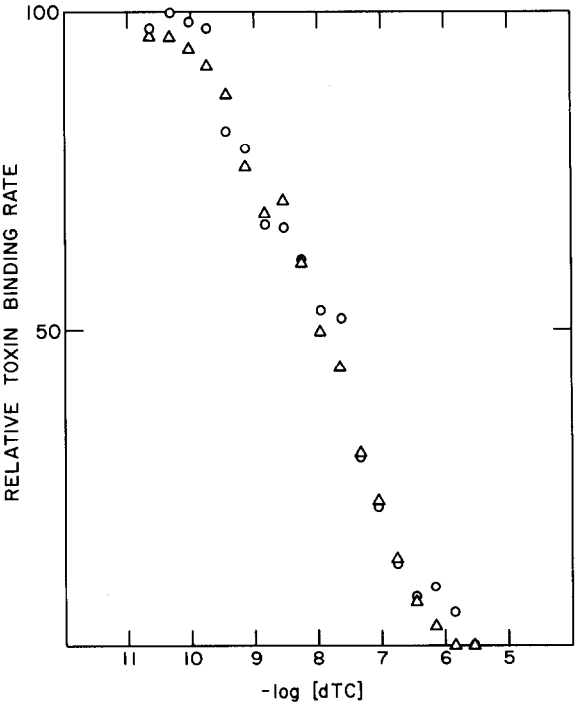

Fig.2. Effect of d-tubocurarine on the rate of binding of α-Bgt to receptor from normal and denervated diaphragm. Serial dilutions of the drug were prepared and divided into two sets of tubes. Aliquots of extracts from denervated (o—o—o) and control (△—△—△) diaphragms were then added and incubated with the ligand for about 10 min at room temperature. The binding reaction was started by the addition of toxin. The concentrations of receptor, toxin and drug during the reaction were 2×10^{-11} M, 4×10^{-11} M, and as indicated on the abscissa, respectively; buffer composition was 10 mM sodium phosphate pH 7.4, 0.1% Triton X-100, 0.02% sodium azide. Receptor–toxin complex formation was allowed to proceed for 10 min, to about 40% of completion. Data were corrected for background and normalized to binding rates seen in the absence of d-tubocurarine.

a 50-fold increase is seen. Extracts from normal diaphragms and from diaphragms that had been denervated for two weeks were investigated for ligand binding affinities by the toxin binding rate assay. Results from a d-tubocurarine experiment are shown in fig. 2; additional data are compiled in table 1. No distinction is possible between the two types of receptor as determined by their affinity for any of the ligands tested.

The biochemical comparison of normal and denervation-induced AcChR from muscle is rendered difficult by the extreme sparsity of these molecules. What little information is available at present has been obtained by means of neurotoxins of high specific radioactivity. Thus the amount and distribution of AcChR have been measured in healthy muscle and at various intervals after denervation [3,4,7,8] and estimates of molecular parameters, such as radius of gyration and sedimentation coefficient, of detergent-solubilized receptor–toxin complexes have been obtained [4,5]. Little is known about the drug binding properties of solubilized receptors from muscle. Radioactive neurotoxins can be utilized to study the interaction of ligands with very small quantities of nicotinic receptors. Assuming that toxin and drug compete for the same site, toxin binding rate is reduced to half when the concentration of the competing drug equals its dissociation constant, i.e., occupies half of the binding sites. The identity of 'protection constants,' obtained by toxin rate studies, and dissociation constants measured by equilibrium dialysis, has been demonstrated by Weber and Changeux [9].

Our failure to detect pharmacological differences between the normal and denervation-induced AcChR may be taken as evidence for the structural similarity of their binding sites, and perhaps of the receptor molecules as a whole. It should be pointed out however that the results appear to contradict some earlier studies on

Table 1
Comparison of ligand affinities for normal and denervation-induced receptor molecules.

Drug	Number of experiments	$-\log K_p$ normal	Denervated
d-Tubocurarine	3	7.90	7.95
Acetyl Choline	4	6.60	6.70
Decamethonium	3	6.26	6.21
Carbamyl Choline	2	5.74	5.59
Hexamethonium	2	4.28	4.20
Nicotine	1	6.30	6.30
Flaxedil	1	7.05	7.00
Succinyl Choline	1	6.55	6.35

The effect of various ligands on the rate of α-Bgt binding was investigated as described in the legend to fig. 2; acetylcholine experiments were carried out in the presence of 10^{-3} M eserine. Ligand affinities are related to a 'protection constant' K_p defined as that concentration of a drug at which toxin binding rate is reduced to half of the value seen in the absence of drugs. Mean values are given in cases where K_p was determined more than once.

receptor–drug interaction. Beránek and Vyskočil, using electrophysiological techniques [10] and Waser, employing autoradiography [11] on the intact organ, noticed reduced d-tubocurarine sensitivity in denervation-induced receptors. To explain this discrepancy one may assume that the function of the AcChR, including its pharmacological properties, depends on how the molecule is integrated into the membrane. It is not altogether unlikely that intrasynaptic and extrasynaptic and extrasynaptic receptors differ in the way they are built into membrane, the former being arranged in stable lattice-like structures, as recently observed in the postsynaptic membrane of *Torpedo marmorata* [12], the latter perhaps more loosely inserted into the sarcolemma [7] and undergoing more rapid turnover [13]. When Feltz and Mallart observed that the ionic selectivities of the channels associated with normal and denervation-induced receptors differ [14] they suggested that different properties might arise from either a difference in nature or a difference in the spatial arrangement of the receptor molecules. The data reported in the present communication favor the latter view.

Acknowledgments

We thank Miss Mercedes Diaz for technical help and Dr M. A. Raftery for assorted cholinergic drugs. This study was supported by a Biomedical Sciences Support Grant from NIH and an award from the Research Foundation of the State University of New York.

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