Acetylcholine Receptors

Revised Estimates of Extrajunctional Receptor Density in Denervated Rat Diaphragm

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A B S T R A C T   The number of extrajunctional acetylcholine receptors (^{125}I-labeled α-bungarotoxin binding sites) per unit length of muscle fiber and the average fiber circumference were determined for rat diaphragm muscle fibers denervated 0, 2, 4, 7, 10, and 14 days. From these data receptor densities (sites per square micrometer of surface) were calculated. Values thus obtained were considerably lower than those estimated previously by autoradiography. Receptor density increased from < 6 sites/μm^2 in innervated muscle to 635 ± 29 sites/μm^2 14 days after denervation. The *form* of the relationship between receptor density and acetylcholine sensitivity and the time-course of change in receptor density after denervation are as previously reported.

INTRODUCTION

Using ^{125}I-labeled α-bungarotoxin and autoradiography, Hartzell and Fambrough (1) reported upon the distribution of acetylcholine receptors (α-bungarotoxin binding sites) in denervated rat diaphragm, the number of binding sites per unit area of surface membrane (receptor density), and the relation between these values and corresponding acetylcholine sensitivities determined by iontophoresis. The calculations of receptor density involved two assumptions which, in the light of results presented below, were probably incorrect: that the efficiency of grain production was 0.5 grains per disintegration during radioautography of skeletal muscle fibers to which ^{125}I-labeled α-bungarotoxin is bound and that specimen preparation did not alter surface area significantly. Using different techniques, I have obtained substantially lower values for receptor density. The best possible values of acetylcholine receptor density in denervated muscle membranes are needed for comparison with data from freeze-fracture studies of these membranes, for a quantitative picture of receptor function in relation to acetylcholine "noise" studies and as a basis for interpreting chemosensitivity measurements.

METHODS

Isolation and Iodination of α-Bungarotoxin

α-Bungarotoxin was isolated from the venom of *Bungarus multicinctus*, obtained from the Miami Serpentarium, and was purified and characterized as previously described (1, 2, 3). The α-bungarotoxin was iodinated by the chloramine-T method (4) and the iodinated α-bungarotoxin purified and its specific activity determined as previously described (1, 3). The specific activity ranged between 1.5 and 4×10^4 Ci/M for different experiments.

Binding of α-Bungarotoxin to Muscle

Male rats (273–340 g, Sprague Dawley) were used in these experiments. Left hemi-diaphragms of control rats or rats denervated 2–14 days were pinned to stainless steel grids and a layer of deep muscle fibers was exposed by dissecting along the phrenic nerve as it courses through the muscle and then carefully peeling and dissecting back the cut fibers for several millimeters. The pinned diaphragms were incubated for 3 h at 37°C in a modified Ham's F-12 culture medium (5) buffered with N-2-hydroxy-ethyl piperazine-N′-2-ethane sulfonate as zwitterion and containing 0.5 % bovine serum albumin and 0.5 μg/ml iodinated α-bungarotoxin. The diaphragms were washed with 12 5-min rinses in cold medium lacking α-bungarotoxin, followed by an overnight rinse in medium at 4°C. Diaphragms were then fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer pH 7.4, rinsed, and stained for acetylcholinesterase by the Karnovsky-Roots method (6) to reveal end plates.

Determination of α-Bungarotoxin Binding Sites Per Unit Length of Muscle Fiber

After glutaraldehyde fixation, fibers were rigid and easily teased into small bundles. End plate-free segments of undamaged muscle fibers were dissected out and trimmed to 2.0 mm. All samples were taken from the area 1–4 mm toward the central tendon from the phrenic nerve and a few millimeters ventral to the site at which the phrenic nerve enters the diaphragm. The bundles of fiber segments were then placed on glass microscope slides, crushed under a coverslip, and the number of fibers in each bundle counted at ×125 or ×500 magnification.

Each bundle of fibers was then transferred to a glass tube (6 × 50 mm). Ten μl of 70 % nitric acid were added, each tube was sealed, and the sample hydrolyzed at 100°C for 15 min. Each hydrolysis tube was cut open and 100 μl of 1 % Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) in 0.1 M Tris-HCl buffer, pH 7.6, was added. Then the entire tube and contents were placed in a scintillation vial with 1 ml of 1 % Triton X-100 solution and 10 ml of Triton-toluene scintillation cocktail (2.8 g PPO (2,5-diphenyloxazole), 0.035 g POPOP (1,4-bis[2-(5 phenyloxazolyl)-benzene]), 665 ml toluene, and 333 ml of Triton X-100/liter). Samples were counted at 60 % efficiency in a scintillation spectrometer. The number of α-bungarotoxin binding sites per unit length of muscle fiber was then calculated for each sample.

Measurement of Muscle Fiber Circumference

Male rats (302–333 g) were fixed by whole body perfusion through the left ventricle. Rats were anaesthetized with Diabutal, then perfused with fresh Tyrode solution (pH 7.2) containing 0.01 % heparin and 0.1 % procaine for 1–3 min and then with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), administered through the same cannula at a rate of approximately 20–40 ml/min. Then the left hemidiaphragm, bathed in fixative, was dissected free and fixed overnight in cold fixative. Portions of diaphragm (from the same region as those used in estimation of α-bungarotoxin binding sites per unit length of fiber) were postfixed in 1 % osmium tetroxide, dehydrated in an ethanol series and propylene oxide, and embedded in medium-soft Spurr plastic (128.8 ml nonenyl succinic anhydride, 28 ml diglycidyl ether of polypropyleneglycol, 46.5 ml vinylcyclohexene dioxide, and 4 ml dimethyl amino ethanol) (7). Cross sections 1 and 2 μm in thickness were cut with glass knives on a Porter-Blum MT-2B ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.) and mounted on glass slides. Sections were photographed at X200 magnification with a Zeiss phase-contrast microscope (Carl Zeiss, Inc., New York, N. Y.). Photographs of a stage micrometer grid were also made and pictures were printed at approximately X800 total magnification. Montages representing cross sections of approximately
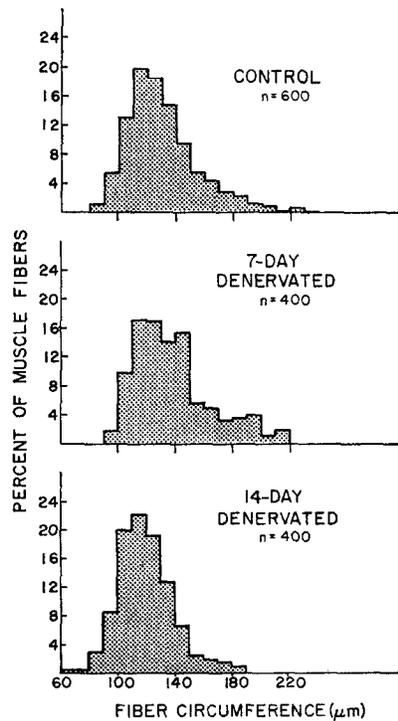

FIGURE 1. Histograms of muscle fiber circumferences in control and 7- and 14-day denervated rat diaphragms.

400 fibers were constructed, and the circumferences of 50–200 fibers were measured using an electronic graphics calculator (Numonics Corp.).

RESULTS AND DISCUSSION

Rat diaphragm undergoes a transient hypertrophy after denervation. As a result average fiber circumference increases slightly in the first few post-denervation days and histograms of fiber circumferences reveal an increased population of larger caliber fibers (Fig. 1). By 14 days postdenervation, significant fiber atrophy has begun. However, changes in fiber surface area during this period are only about ±10% of control values and are thus actually of less importance in calculation of receptor site density than I had imagined. During these 2 wk, extrajunctional acetylcholine sensitivity and the number of α-bungarotoxin binding sites per square micrometer of muscle surface increase from undetectable or scarcely detectable levels to approximately their maximal values (Table I).

The values for α-bungarotoxin binding site density in Table I are 60–75% lower than those estimated previously by autoradiography (i.e., up to 1,695/μm²). I have made photographs of single muscle fibers just after teasing from glutaraldehyde-fixed muscle and again after autoradiography. There appears to be no change in sarcomere length but some decrease in apparent fiber diameter due to dehydration. This shrinkage is at least partially responsible

TABLE I

MUSCLE FIBER CIRCUMFERENCE, EXTRAJUNCTIONAL
ACETYLCHOLINE RECEPTOR DENSITY, AND
ACETYLCHOLINE SENSITIVITY OF DENERVATED
RAT DIAPHRAGM

Days denervated	Muscle fiber circumference (mean)		Acetylcholine receptor density (α-bungarotoxin binding sites/square micrometer muscle surface) (± SEM)		Acetylcholine sensitivity (± SEM)
	μms				*mV/ncoul*
0	129.8	(600)*	<6±2	(10)‡	<0.01
2	141.9	(200)	13±2	(10)	<0.01
4	135.8	(200)	142±12	(20)	37±9
7	135.7	(400)	302±19	(17)	112±13
10	150.5	(50)	502±25	(20)	189±18
14	120.1	(400)	635±29	(32)	310±36

Muscle fiber circumferences and α-bungarotoxin binding site densities were determined as described in Methods.
Values for acetylcholine sensitivity are taken from reference 1.
* Numbers of fibers measured. 50–200 fibers were measured per rat. Distributions for 0-, 7- and 14-day denervated fiber circumferences are shown in Fig. 1.
‡ Number of samples. Seven to 10 samples were analyzed per rat.

for the larger estimates of receptor density. Another probable source of error was the assumption of 0.5 grains/decay in the radioautographs. These were radioautographs of whole-mounted muscle fibers of sufficient thickness that it was not possible to control emulsion thickness immediately around the specimen.[125]I decay produces moderately powerful β-particles (8) which can generate multiple grains when penetrating thick emulsion, again leading to an overestimate of binding sites. The present values of α-bungarotoxin binding site density (Table I) were obtained by methods which should minimize errors in estimation of surface area and allow direct measurement of the number of α-bungarotoxin molecules bound in each sample.

A nonlinear relationship between acetylcholine receptor density (α-bungarotoxin binding sites per square micrometer) and acetylcholine sensitivity reported previously (1) still obtains with the revised estimates of receptor density. Plotting log acetylcholine receptor density vs. log acetylcholine sensitivity gives a straight line with slope 0.62. In reference 1 we plotted log acetylcholine receptor density vs. reciprocal of acetylcholine sensitivity so that the slope, −0.53, was negative. An interpretation of this relationship was offered previously (1).

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