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Acetylcholine receptor density and binding in murine dystrophy

Theo P.A. Kruck and David M. Logan

Department of Biology, York University, Downsview, Ontario (Canada)

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The concentration and binding characteristics of acetylcholine receptors (AChR) in sarcolemmal fractions from normal and dystrophic mice have been measured. Unlike the results following denervation, AChR concentration (per g wet weight) is unchanged and AChR affinity for two different α-neurotoxins is also unchanged. In contrast sarcolemmal ATPase is significantly reduced in dystrophic tissues. These data reaffirm that denervation is a poor model of dystrophy.

Mice, homozygous for the 'dystrophic' mutation (dy/dy) express a wide range of physical and biochemical difference compared to normal. These include, altered membrane characteristics [1], lipid and protein changes [2], altered muscle fibre distributions [3] and changes in nerve structure and activity [4]. This very diversity of effects makes identification of the primary lesion extremely difficult. Particular attention, however, has been directed to membrane changes and altered neuromuscular interaction (possibly trophic effects). A critical component of both foci is the sarcolemmal acetylcholine receptor (AChR) where changes in density, distribution or binding characteristics could contribute to the observed tissue changes. To assess possible changes in AChRs in murine dystrophy, we have measured receptor density and binding characteristics in normal and dystrophic muscle membranes. For comparison we have also measured the concentration of a membrane specific ATPase. The data indicate that AChR changes are unlikely to underlie the diverse changes observed.

Correspondence: D.M. Logan, Department of Biology, York University, Downsview, Ontario, Canada, M3J 1P3.

Animals. Female ReJ129 dy/dy mice and phenotypically normal littermate controls were obtained from Jackson laboratories. The experiments reported here were all performed on animals about 75 days old.

Chemicals. ^{125}I-α-Bungarotoxin (^{125}I-α-Bgt) (initial activity 130 Ci/mmol) was obtained from New England Nucleare. Crude venoms from *Bungarusfasciatus* and *Naja naja siamensis* and human serum albumin were obtained from Sigma Chemical Company. All other chemicals were obtained from standard suppliers and reagent grades were used throughout.

Preparation of neurotoxin C. α-Neurotoxin C which competes with α-bungarotoxin for AChR binding was prepared as described by Kruck and Logan [5].

Acetylcholine receptor preparation. Sarcolemmal membrane vesicle fraction were isolated from mouse hind limb muscles as described by Jones et al. [6]. Receptors were freed from the membrane vesicle fraction by the addition of Triton X-100 to a final concentration of 2%. After 30 to 60 min incubation, the preparation was transferred to silanized glass tubes (4°C) for use the same day (in affinity studies) or stored at −80°C.

α-Bungarotoxin binding assay. The binding of ^{125}I-α-bungarotoxin to AChR was measured in reaction mixtures containing solubilized AChR preparations in buffer A (10 mM K_2HPO_4/KH_2PO_4 (pH 7.4), 30 mM NaCl, 2% Triton X-100 and 1 mg/ml human serum albumin) to which were added ^{125}I-α-bungarotoxin and additional buffer to give a usual final volume of 250 μl. The binding reaction was allowed to proceed for an appropriate time (usually 30 min) at room temperature and the reaction was then terminated by the addition of 50 μl of buffer containing 1 mg/ml crude venom from *Naja naja siamensis* (buffer B). The resulting solution was then applied to a DEAE filter which had been previously soaked with buffer B and allowed to flow through by gravity. After 10 min the filters were washed with 100 μl of buffer B (under gravity). When the buffer had washed through the filter, vacuum was reapplied and the filter was further washed in order with three 5 ml volumes of buffer (minus human serum albumin and venom), 5 ml H_2O and 5 ml of methanol. The filters were then heated to 90–100 °C until completely dry and the dried filters were counted in 10 ml of PPO/POPOP toluene scintillation fluid by standard techniques. In each case the data were corrected for non specific α-bungarotoxin binding to the filter. Under these conditions binding equilibrium is reached in approx. 15 min or less.

The maximum binding of α-bungarotoxin, measured when ^{125}I-α-bungarotoxin (of known specific activity) is present in excess, is used to calculate the concentration of available AChRs. The affinity of AChRs for α-bungarotoxin is measured in a series of equivalent assays. In these cases, however, a series of initial concentrations of α-bungarotoxin varying from well below saturation to saturation is tested. In each case the bound and free toxin concentrations are calculated and the ratio bound/free is then plotted against bound toxin in a Scatchard plot. The slope of this plot provides the affinity constant.

α-Bungarotoxin Binding in Competition with Neurotoxin C. Fresh membrane vesicle preparations were diluted in buffer A and divided into aliquots of 50 or 100 μl containing $(1–2) \cdot 10^{-15}$ moles of ^{125}I-α-bungarotoxin binding sites each in 1.5 ml polyethylene 'Eppendorf' tubes. Various amounts of α-neurotoxin C were added followed by 10 μl of $2 \cdot 10^{-9}$ M ^{125}I-α-bungarotoxin freshly purified stock solution and the volume was adjusted to 200 μl with buffer A. The binding was allowed to proceed for 30 min and was then stopped by addition of 50 μl of buffer B. The reaction mixture was then immediately analyzed for ^{125}I-α-Bgt/AChR complex formed in the presence of different amounts of C. From this data the affinity for neurotoxin C was calculated.

$(Na^+ + K^+)$-ATPase assay. $(Na^+ + K^+)$-stimulated ATPase was determined in previously frozen membrane vesicle preparations. Proteins contained in the membrane vesicles, including the plasma membrane 'marker' enzyme $(Na^+ + K^+)$-ATPase were freed from the membrane matrix and solubilized by the addition of Triton X-100 to a final concentration of 2%. ATPase activity was determined according to the method of Philipson and Edelman [7]. The reaction mixture contained 10–50 μl membrane vesicle extract, 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM Tris, pH 7.4 in a final volume of 1.0 ml. The reaction mixtures were brought to 37 °C in a shaking water bath and equilibrated for 2 min. 100 μl of a solution, containing 30 mM Na_2 ATP in 50 mM Tris buffer (pH 7.5), were then added to initiate the reaction. After 30 min, the reaction was stopped by the addition of 1 ml of 2% SDS, 50 mM Tris buffer (pH 7.4), and analyzed for liberated monophosphate. The ouabain-inhibitable fraction of the ATPase activity was measured using duplicate samples, adding ouabain to one set to give a final concentration of 1 mM. The $(Na^+ + K^+)$-ATPase activity (Na^+ pump) was taken as that part of the total phosphate production that was ouabain inhibitable. All activities were expressed in mmol P_i/g protein per hour.

Computational methods. Data points were fitted by straight lines using the SAS statistics software package at York University which provides a least-squares regression, computes mean slopes and tests for equivalence or non-equivalence. Standard errors for all data points, slopes and intercepts were also calculated. The resulting regression lines were then used in a Scatchard analysis to determine affinity constants in normal and dystrophic tissues.

$(Na^+ + K^+)$-ATPase activity. This enzyme is reported to be a 'marker' of sarcolemma and is

TABLE I

ATPase ACTIVITY IN NORMAL AND DYSTROPHIC SARCOLEMMAL VESICLES

ATPase activity is measured as mmol P_i/g protein per hour ± S.E. where n is the number of preparations assayed. Ouabain-inhibitable ATPase is the percent of total ATPase which is inhibitable by an excess of ouabain.

	Tissue		Protein (mg/g wet wt.)	ATPase activity
Total ATPase	Normal	($n = 4$)	1.08	40.12 ± 2.00
	dystrophic	($n = 4$)	0.80	18.9 ± 7.02
Ouabain-inhibitable ATPase	normal	($n = 4$)		42.5%
	dystrophic	($n = 4$)		33.3%

thus a useful indicator of the changes of a nonreceptor protein in dystrophic sarcolemma. The concentration of ATPase in normal and dystrophic sarcolemmal fractions is shown in Table I. Two types of assay are presented. In the first the ATPase concentration (per gram of tissue) is shown to be decreased by 63% in dystrophic sarcolemma compared to normal. The second set of assays shows the proportion of ATPase activity in each tissue which is inhibitable by ouabain. In normal tissue 42.5% is inhibitable while in dystrophic only 33.5% is inhibitable.

Acetylcholine receptor density and binding. The concentrations of AChRs in normal and dystrophic sarcolemma are reported in Table II. While the number per limb is reduced, this probably reflects atrophy of the muscle since the concentration (based on wet weight) is unchanged or increased (based on protein). Thus, in contrast to ATPase, the AChR concentration is retained in dystrophic tissue.

There is a possibility, however, that, although undiminished in concentration, other AChR characteristics are changed. To test this we examined two binding parameters, the affinity for α-bungarotoxin and the inhibition of α-bungarotoxin binding by a completing neurotoxin. The assays in both cases involve measurement of toxin binding at different inputs of toxin and the construction of Scatchard plots in each case from which affinity constants are determined. In every case the plots were linear with no evidence of curvature (and hence there is no evidence in these assays of cooperativity in the binding). The data obtained are shown in Table II. The affinity of dystrophic receptors for α-bungarotoxin is unchanged from that in normal tissue. Further when α-bungarotoxin binding is measured in the presence of α-neurotoxin C, the calculated affinity for C is also unchanged. The receptors are thus unchanged with respect to their binding to two strong ligands.

Discussion. Murine dystrophy is a useful model of the human disease with genetically determined nerve, membrane and muscle changes which develop in a regular time sequence in young mice. Among these changes particular attention has focussed on membrane alterations because of the current perception that membrane modification is probably the primary pathogenic lesion. Both

TABLE II

AChR CONCENTRATION AND BINDING IN NORMAL AND DYSTROPHIC SARCOLEMMA

Values are given ± S.E. where n is the number of preparations assayed.

	Tissue		Per hind limb ($M \times 10^{-14}$)	Per g wet wt. ($M \times 10^{-14}$)	Per g vesicle protein ($M \times 10^{-11}$)
AChR concentration	normal	($n = 9$)	3.59 ± 0.19	5.13 ± 0.36	4.85 ± 0.34
	dystrophic	($n = 9$)	1.50 ± 0.11	5.22 ± 0.28	6.53 ± 0.35
			α-Bgt affinity ($M^{-1} \times 10^{-9}$)		α-Neurotoxin C affinity ($M^{-1} \times 10^{-8}$)
AChR binding	normal	($n = 7$)	1.03 ± 0.27		1.44 ± 0.10
	dystrophic	($n = 7$)	1.07 ± 0.17		1.47 ± 0.10

membrane lipids and proteins have been studied, often with contradictory results, although many of the differences can now be ascribed to age, sex or strain differences. In our current work we were concerned with AChRs in murine dystrophy because of their central role. Harris and Ribchester [8] reported that the morphology and physiology of nerve terminals in dystrophic 129ReJ mice are apparently normal but did not measure AChR density or binding directly. Such measurements have been made in another strain however, C57B1/6J [9]. In this strain the AChR concentration appears normal in adult mice and the receptor itself is apparently unchanged with respect to sedimentation and subunit composition although the specific binding was not measured. Surprisingly, cultured muscle cells of this strain exhibit lower ACh sensitivity and reduced AChR concentration apparently because of increased endocytosis [10]. Our data show that AChR concentration is not diminished in ReJ 129 dystrophic mice but is conserved at the expense of other proteins such that the concentration, based upon total protein is actually increased. Further, the binding of the receptor for two different strong ligands is unchanged from normal, i.e. the appearance of high-affinity AChRs which occurs following denervation does not occur in dystrophy. Clearly denervation, which leads to an increased content of receptors which themselves show an increased affinity for α-bungarotoxin is a poor model of dystrophy.

In contrast, a second membrane protein ($Na^+ + K^+$)-ATPase is clearly reduced in dystrophy. A similar observation was reported by Bray et al. [11], however, Abdel-Aziz et al. [12] studying C57B1 mice demonstrated that such changes are age and muscle specific. Thus ouabain binding sites are increased in EDL muscle but decreased in soleus with the difference in the EDL vanishing by 14–16 months while the soleus changes are retained. The data obtained reaffirm the necessity of specifying the age, strain and fiber composition of muscles studied in any comparison between normal and dystrophic tissue.

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