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PHARMACOLOGICAL ASPECTS OF NEUROMUSCULAR TRANSMISSION IN THE ISOLATED DIAPHRAGM OF THE DYSTROPHIC (Rej 129) MOUSE

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1 Some aspects of the pharmacology of neuromuscular transmission have been studied in the isolated diaphragm of the normal and dystrophic mouse.

2 The effects of (+)-tubocurarine and atropine on the indirectly elicited twitch responses of the dystrophic diaphragm were indistinguishable from normal.

3 Intracellular recording techniques revealed no significant differences between the rise time, time to half decay, frequency and amplitude of miniature endplate potentials (m.e.p.ps) recorded in dystrophic muscle fibres, compared to those recorded in normal muscle fibres.

4 Transmitter null potential, the size of the available store of transmitter, the probability of release of the transmitter, and the characteristics of endplate potentials (e.p.ps) of dystrophic muscle fibres did not differ from normal.

5 The quantum contents of e.p.ps generated in response to nerve stimulation of 0.1 to 100 Hz were consistently larger in dystrophic muscle fibres than in normal muscle fibres, but the differences were not statistically significant under the conditions of the experiment.

Introduction

Murine muscular dystrophy was described by Michelson, Russell & Harman in 1955. Animals suffering from the disease may be distinguished from their unaffected littermates at the age of about 3 weeks *post partum* by virtue of their small body size and the characteristic flexion of the hind limbs. Michelson *et al.*, (1955) classified the disease as a genetically-determined primary disease of skeletal muscle with an autosomal-recessive mode of inheritance. Although it is now clear that many other tissues and organs such as the peripheral nervous system (Bradley & Jarôs, 1979), the thymus (Karmali & Horrobin, 1976) and the endocrine pancreas (Lundquist, Håkanson, Harris, Libelius & Sundler, 1979) exhibit abnormalities in development, morphology or function, it is still the pathophysiology of the skeletal muscle that claims most attention.

The abnormalities of skeletal muscle are well recognized, and include a reduction in muscle fibre number, variation in muscle fibre diameter, coagulation and segmental necrosis in individual muscle fibres, and central nucleation (See, for example, West & Murphy, 1960; Rowe & Goldspink, 1969). Dystrophic muscles are weaker than normal, both in absolute terms and when expressed in terms of tension generated per unit wet weight or per unit area, and the rate of shortening per sarcomere is reduced (Douglas & Baskin, 1971; Taylor, Fowler, Mason & Spann, 1971; Harris & Wilson, 1971). It has also been claimed that 'functional' denervation of muscle fibres may contribute to the observed weakness (McComas & Mrożek, 1967; Law, Atwood & McComas, 1977), a claim that is still controversial (see Harris & Ribchester, 1979). However, in spite of the obvious importance of this controversy, the role of the neuromuscular junction in the expression of murine muscular dystrophy has only rarely been systematically examined, and there are few reports on either the physiological or pharmacological behaviour of the neuromuscular junction in the muscles of the dystrophic mouse.

In the earliest work on pharmacological aspects of transmission apparently available, Baker, Wilson, Oldendorf & Blahd (1960) reported that intact dystrophic mice were relatively insensitive to injections of tubocurarine. Later experiments on isolated nerve-muscle preparations (Baker & Sabawala, 1963; Beaulnes, Bois & Carle, 1965) seemed to confirm these early observations, and led to the concept that murine muscular dystrophy was associated with either some form of abnormality that led to the formation of an increased number of acetylcholine receptors on the postsynaptic membrane (Baker & Sabawala, 1963), or with an abnormality, presynaptic in

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origin, that led to an increase in the amount of 'acetylcholine or acetylcholine-like' material (Beaulnes *et al.*, 1966) released from the nerve terminal in response to a nervous impulse.

In view of the rather imprecise nature of these early pieces of work, and of the continuing controversy surrounding the role of the neuromuscular junction in the aetiology and/or expression of the disease, we have re-examined some pharmacological aspects of neuromuscular transmission in isolated nerve-muscle preparations of the dystrophic mouse using both 'classical' and electrophysiological techniques. A preliminary account of some of these experiments has been presented to the Physiological Society and to the New York Academy of Sciences (Harris & Ribchester, 1976; 1979, respectively).

Methods

The experiments were carried out on nerve-muscle preparations removed from dystrophic mice of either sex of the Bar Harbor 129 Rej strain and their clinically normal litter mates. The mice were bred in the Muscular Dystrophy Group Laboratories from stock originally obtained from the Jackson Laboratories, Bar Harbor, Maine. The animals were killed when aged 3 to 6 months.

Bathing fluids

The isolated preparations were bathed in Liley's fluid (Liley, 1956) of the following composition (mM): K^+ 5.0, Na^+ 150, Ca^{2+} 2.0, Mg^{2+} 1.0, Cl^- 148, $H_2PO_4^-$ 1.0, HCO_3^- 12.0 and glucose 11.0. The solution was maintained at room temperature and equilibrated with 5% CO_2 and 95% O_2. Cut-fibre preparations (q.v) were usually maintained in 2.5 mM K^+ rather than in 5.0 mM (see Hubbard & Wilson, 1973).

Experiments utilizing the twitch response

These experiments were carried out on intact hemidiaphragm preparations. The phrenic nerve was stimulated at 0.1 Hz with pulses of 0.05 ms duration and supramaximal voltage. The contractions were recorded isometrically with a Devices Ltd. dynamometer (UFI), amplifier (3559; HF cut 150 Hz) and recorder (MXZ). The resting tension on the muscle was adjusted until the twitch response was maximal. The mean ($\pm$s.e.mean) resting tension required was 1.2 ± 0.09 g ($n = 16$) for normal muscles and 1.1 ± 0.18 g ($n = 13$) for dystrophic muscles. At the end of every experiment, the preparation was removed, the rib-cage trimmed away and the muscle lightly blotted and weighed. The recording system was then calibrated.

The effects of (+)-tubocurarine and atropine on the twitch response were measured by constructing dose-response curves. A contact time of 2 min was used, followed by a period of washing of 15 min during which nerve stimulation was interrupted. In 6 experiments dose-response curves were constructed to both atropine and (+)-tubocurarine on the same preparation. In order to prevent any bias in these experiments, atropine was used first in 3 experiments and (+)-tubocurarine was used first in the other 3. In all cases, preparations were washed for 1 h between the two parts of the experiment.

The effect of Mg^{2+} on the twitch response was determined by constructing cumulative dose-response curves. The preparation was allowed to equilibrate for 5 min before a change in concentration was made.

Electrophysiological recording methods

The electrical responses of muscle fibres were recorded with glass microelectrodes filled with 3 M KCl. The electrodes had tip potentials <5 mV and resistances of 5 to 15 MΩ. When it was necessary to measure 'input resistance,' 'time constant' or to cause local changes in membrane potential, a second, current passing, electrode was inserted into a muscle fibre 50 to 100 μm away from the recording electrode. The recording and current generating circuits used were home-built, and have been described by Allan, Gascoigne, Ludlow & Smith (1977).

Miniature endplate potentials

Miniature endplate potentials (m.e.p.ps) were recorded from intact hemidiaphragm preparations, pinned to small Sylgard strips (Dow-Corning Ltd.) and immersed in Liley's fluid (Liley, 1956) at room temperature. The presence of m.e.p.ps with a rise time of <1.1 ms was taken to indicate the focal location of the endplate. The m.e.p.ps were recorded on an FM tape recorder at 3.75 i.p.s. (Precision Instruments PI 6200) for later analysis. The 'input resistance' and 'time constant' of the muscle fibre were routinely recorded.

Endplate potentials

Endplate potentials (e.p.ps) were recorded from cut fibre preparations (Barstad, 1962). The muscle fibres were cut 1 to 2 mm either side of the zone of innervation and the preparation left for 20 to 40 min. During this time, the resting potential fell from a mean of approximately -70 mV to a mean of approximately -15 mV. The final resting potential of a cut fibre varies between -10 and -40 mV. During these experiments, therefore, a second, current-passing electrode was used to maintain the membrane potential

at −20 mV at the endplate region. At this level of membrane potential, sodium inactivation is virtually complete, and it is possible to record endplate potentials uncomplicated by any form of regenerative response.

Quantum content of e.p.ps

The quantum content (m) of e.p.ps was estimated by the variance method (del Castillo & Katz, 1954; Martin, 1955). This method assumes that the release of transmitter at undepressed junctions obeys Poisson rather than binomial statistics. The assumption has been tested and considered valid by Martin (1966) and by Elmqvist & Quastel (1965). However, Miyamoto (1975) has suggested that the release is best described by binomial statistics at low rates of stimulation and Wilson (1977) considers only binomial statistics to be accurate. Clearly, the statistical nature of the release process is controversial. However, in spite of the difficulties involved in the use of the variance method, it does allow a fairly detailed study of some aspects of presynaptic activity to be made. Endplate potentials were considered focal if they had a rise time <1.1 ms. Trains of 20 e.p.ps were recorded at 0.1 and 1.0 Hz, and trains of 40 e.p.ps were recorded at 10, 30 and 100 Hz. An interval of 1 min was allowed between each period of stimulation. The e.p.ps were recorded on moving film and measured, after suitable enlargement, to the nearest 0.05 mV. Quantum size and mean quantum content were then calculated after correcting e.p.p. amplitudes for the non-linear summation of quantal units (Martin, 1955). In order to avoid early tetanic rundown and the regression of e.p.p. amplitude that occurs at high frequencies of stimulation (Elmqvist & Quastel, 1965), only the last 20 e.p.ps of the train of 40 generated at frequencies in excess of 1 Hz were used in the appropriate calculations.

Theoretically, the variance method for the calculation of quantum content underestimates the true quantum content if corrections for variation in noise and quantum size are ignored. In practice, the contribution of noise in the recording system is negligible. Moreover, with cut fibre preparations, where m.e.p.ps cannot be recorded, it is not possible to make a direct measurement of the within-fibre variance in quantum size. This correction was not, therefore, applied. However, if it is assumed that the within-fibre variance in m.e.p.p. amplitude recorded in intact preparations (see Results, page 415) is the same as the within-fibre variance in quantum size in cut fibres, it is possible to estimate the size of the introduced error from the formula:

$$m_{cor} = m_{cal}\,(1 + CV^2_{mepp})$$

where m_{cor} is the corrected quantum content, m_{cal} is the quantum content calculated by the variance method and CV_{mepp} is the coefficient of variation of m.e.p.p. amplitude. Such calculations revealed that the uncorrected values of quantum content are underestimates by approximately 3% and 7% in normal and dystrophic preparations respectively. Errors of this size had little practical significance and were masked by the much greater variations in the calculated quantum contents of e.p.ps in both normal and dystrophic muscles (see Table 4).

Transmitter null potential

The correction for non-linear summation of quantal units involves a term for transmitter null potential. This has been assumed to be between −10 and −12 mV in cut fibre preparations (Hubbard & Wilson, 1973; Wilson, 1977), but our own preliminary results suggested that it was closer to −4 mV (Harris & Ribchester, 1976). In the present experiments the transmitter null potential (t.n.p.) was recorded in every experiment by making stepwise changes in the resting potential (using the current passing electrode) and recording the amplitude of the evoked e.p.p. The resting potential was varied between +30 and −30 mV, allowing e.p.p. amplitude to be plotted as a function of membrane potential. The t.n.p. could be measured directly from the curve and the value thus obtained used in the appropriate calculations for correcting for non-linear summation.

Transmitter store size and the probability of release

The size of the immediately available store, and the probability of release of transmitter were estimated from the early tetanic rundown of e.p.p. amplitude at 100 Hz (Elmqvist & Quastel, 1965). It is assumed that the early tetanic rundown represents the depletion of the available store without replenishment. This commonly involves the first 4 to 10 e.p.ps. Extrapolating the line joining $m\,v\,\Sigma m$ to $m = 0$ gives an estimate of the size of the store of available transmitter, and the ratio of the size of the first e.p.p. to the size of the store gives an estimate of the probability of release. Both estimates are subject to error (Christensen & Martin, 1970), but might be expected to reveal large and systematic differences between normal and dystrophic junctions.

Statistical analysis of results

The results are routinely presented as mean ±s.e.mean. The differences between two means were analysed either by Student's t test or, where the variances differed significantly, by Welch's test (Welch, 1937). A probability level of $P < 0.05$ was considered statistically significant.

Results

Experiments on the twitch response of the isolated diaphragm

General properties The diaphragm of the mouse is often considered to be 'spared' the dystrophic process on the grounds that even severely crippled animals breathe without obvious difficulty. However, the isolated dystrophic hemidiaphragm preparations used in these experiments were lighter than normal, and generated less tension in response to a single stimulus to the nerve than normal. This was true whether the response was expressed in absolute terms or as tension generated per unit wet weight of tissue (Table 1). Weakness is the most characteristic feature of dystrophic muscle, and these results are consistent with previous observations on the mechanical properties of dystrophic muscle *in vitro* and *in vivo* (Sandow & Brust, 1958; Douglas & Baskin, 1971; Taylor *et al.*, 1971; Harris & Wilson, 1971). Histological examination of the diaphragm of the dystrophic mouse revealed fibre-splitting, central nucleation and some variation in muscle fibre diameter (unpublished observation). These morphological abnormalities are typical of dystrophic mouse muscle (Michelson *et al.*, 1955). On the basis of these physiological and morphological criteria, it seems unreasonable to support the view that the diaphragm of the dystrophic mouse is spared the disease process.

Action of (+)-tubocurarine The effect of (+)-tubocurarine on indirectly elicited twitch response of the hemidiaphragm was assessed on 14 normal and 9 dystrophic nerve-muscle preparations. The results (Figure 1) indicate that the sensitivity of the dystrophic preparations is similar to normal.

The curare/atropine ratio Atropine is the classical competitive inhibitor of acetylcholine (ACh) at the 'muscarinic receptor.' At other cholinoceptors, atropine will usually block the effects of ACh, although large concentrations are usually needed. Moreover, there is virtually unequivocal evidence that at non-muscarinic sites, the drug has a non-competitive mode of action (Katz & Miledi, 1973). However, the curare/atropine ratio (that is, the ratio of concentrations of (+)-tubocurarine and atropine respectively needed to induce a 50% inhibition of a given response) has been used as an index of the 'nature' of cholinoceptors (see for example, Itina, 1959; Beranek & Vyskocil, 1967; Harris, Marshal & Wilson, 1973). The mean ratio of 6 dystrophic preparations was 0.0011 ± 0.0004, and in 6 normal preparations the mean ratio was 0.0013 ± 0.0004. The means were not significantly different.

Table 1 Some properties of the isolated diaphragm preparations of normal and dystrophic mice

	Normal	*Dystrophic*
Wet weight	29.6 ± 1.56 (12)	21.8 ± 1.26 (12)*
Resting tension (g)	1.2 ± 0.09 (16)	1.1 ± 0.18 (13)
Twitch tension (g)	7.0 ± 0.41 (16)	3.9 ± 0.36 (13)*
Twitch tension wet weight (g/mg)	0.25 ± 0.018 (12)	0.19 ± 0.016 (13)*

The muscles were stimulated indirectly and the twitch response was recorded isometrically. Resting tension refers to the tension under which the muscles were maintained and at which the muscle response was maximal. Figures are mean ± s.e.mean, and the number of preparations used is given in parentheses.
* Dystrophic significantly different from normal ($P < 0.05$; t test).

Table 2 Some electrical properties of normal and dystrophic diaphragm muscle fibres

	Normal	*Dystrophic*
Resting membrane potential (mV)	−66 ± 0.9 (29)	−66 ± 1.4 (20)
Input resistance (MΩ)	0.54 ± 0.03 (29)	0.55 ± 0.03 (20)
Membrane time constant (ms)	1.97 ± 0.05 (20)	2.00 ± 0.05 (17)

Figures are mean ± s.e.mean, and the number of fibres examined is given in parentheses.

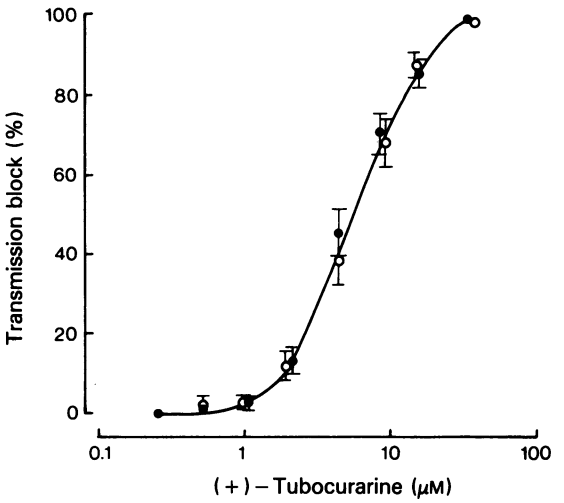

Figure 1 The effect of (+)-tubocurarine on the twitch response of indirectly stimulated diaphragm preparations of normal (●, $n = 14$) and dystrophic (○, $n = 9$) mice. The contact time was 2 min, and doses were repeated every 15 min. The line has been fitted by eye. Vertical lines show s.e. mean.

The effects of Mg^{2+} The effects of Mg^{2+} on neuromuscular transmission were tested by exposing 5 normal and 4 dystrophic nerve-muscle preparations to increasing amounts of the ion. Cumulative dose-response curves were plotted. In both normal and dystrophic preparations, a 50% inhibition of the twitch response was achieved with 10 to 12 mM Mg^{2+} and 100% inhibition was seen with 16 to 20 mM Mg^{2+}. The results on dystrophic muscles were indistinguishable from those on normal muscles.

Experiments with intracellular microelectrodes

General properties of the muscle fibres The mean resting membrane potential, mean input resistance and mean membrane time constant of muscle fibres randomly penetrated in dystrophic muscles did not differ significantly from normal (Table 2).

The spontaneous release of transmitter The spontaneous release of transmitter was studied by measuring the characteristics of the m.e.p.ps in 29 normal and 20 dystrophic muscle fibres. The mean frequency, rise-time, half-decay time and amplitude of the potentials were statistically similar to normal (Table 3). However, there was greater than normal variation in the amplitude of individual m.e.p.ps within a given muscle fibre (see Figure 2). Thus the mean coefficient of variation (that is standard deviation/mean amplitude) of m.e.p.p. amplitude within dystrophic muscle fibres was 0.27 compared with 0.16 within normal muscle fibres.

Transmitter null potential The transmitter null potential (also known as the equilibrium potential) was determined with cut fibre preparations. The amplitude of the e.p.p. was recorded at various membrane potentials as described in Methods. Typical records of e.p.ps recorded under these conditions are shown in Figure 3 and the relationships between amplitude of the end-plate potential and the membrane potential derived from the records shown in Figure 3 are also shown. The mean transmitter null potential of 43 normal fibres was -4.4 ± 0.38 mV, and of 34 dystrophic fibres was -4.8 ± 0.29 mV. The difference was not statistically significant.

The endplate potential The amplitude and time-course of e.p.ps generated in response to a single stimulus were measured in normal and dystrophic

Table 3 Characteristics of miniature endplate potentials (m.e.p.ps) in normal and dystrophic muscle fibre

	Normal	*Dystrophic*
M.e.p.p. amplitude (mV) (corrected to −90 mV)	2.0 ± 0.07 (29)	1.9 ± 0.10 (20)
M.e.p.p. frequency (Hz)	1.4 ± 0.13 (29)	1.6 ± 0.13 (20)
Rise time (ms)	0.7 ± 0.04 (27)	0.7 ± 0.04 (18)
Time to half decay (ms)	1.5 ± 0.04 (27)	1.5 ± 0.08 (18)

Figures are mean ± s.e.mean and the number of fibres examined is given in parentheses. M.e.p.ps were recorded at the resting membrane potential of the muscle fibre, and their amplitudes corrected to a standard resting potential of −90 mV (see Methods).

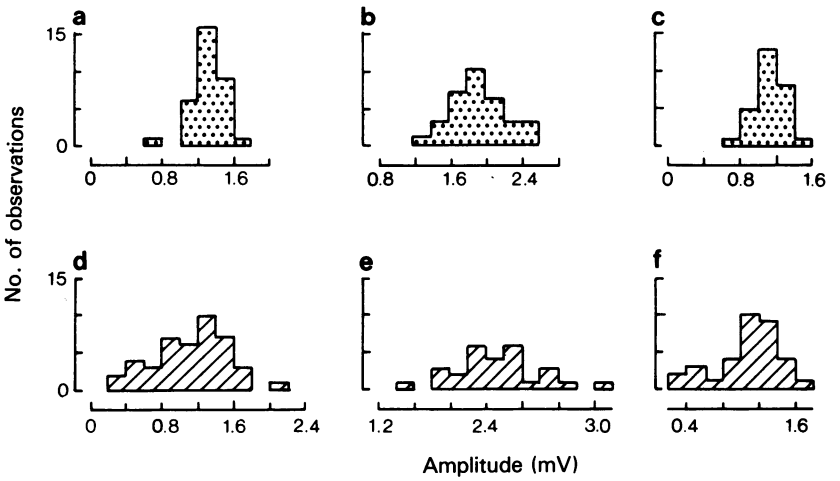

Figure 2 Distribution of the amplitude of miniature endplate potentials (m.e.p.ps) in typical normal (a, b, c) and dystrophic (d, e, f) muscle fibres. Note the wider and skewed distribution of m.e.p.p. amplitude in the dystrophic muscle fibres.

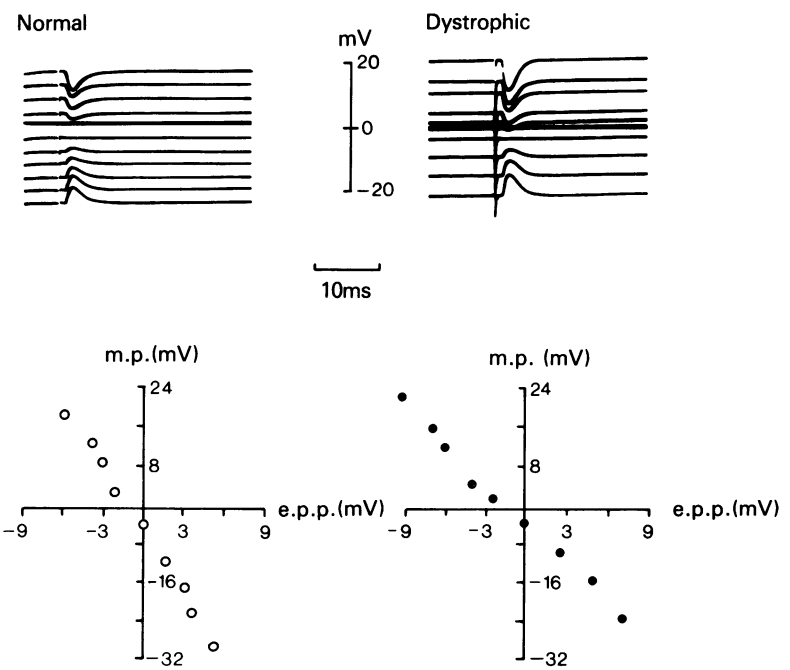

Figure 3 Transmitter null potential in typical normal and dystrophic diaphragm muscle fibres. The muscle fibres were cut, and an e.p.p. was recorded in response to indirect stimulation. The resting membrane potential (m.p.) of the cut fibres was gradually changed using a current passing microelectrode, and successive e.p.ps generated at each level of membrane potential were stored. Amplitude of e.p.p. was then plotted against resting membrane potential, and the null point was taken to be the point of interception on the membrane potential axis.

muscle fibres. In all respects differences between e.p.ps in dystrophic fibres and normal fibres were small and not statistically significant. The mean quantum content of e.p.ps was calculated from measurements of trains of e.p.ps generated at stimulus frequencies of 0.1 to 100 Hz. The results for a number of normal and dystrophic muscle fibres are presented in Table 4. Although the mean quantum content of e.p.ps was considerably higher in dystrophic than in normal fibres at all rates of stimulation used, the differences were not statistically significant ($P > 0.05$; Welch's test).

The effect of long-term stimulation on the end-plate potential Occasionally during the course of an experiment, nerve stimulation suddenly failed to generate an e.p.p. (possibly due to local anoxia, see Krnjević & Miledi, 1959). In a series of experiments designed to test the ability of nerve terminals to liberate transmitter over a relatively long time course, normal and dystrophic cut-fibre preparations were stimulated at 30 Hz for up to 60 s. Out of 22 normal fibres studied, one failed at 20 s, and 2 failed between 30 and 60 s; of 18 dystrophic fibres tested, one failed at 23 s and 1 failed at 51 s. The results indicate that dystrophic nerve-muscle preparations are as 'stable' as normal preparations.

The size of the available store and the probability of release Some typical results showing the relationship between m and Σm are shown in Figure 4. In 14 normal fibres, the estimated size of the available store of transmitter was 2,593 ± 324 quanta, and the probability of release was 0.08 ± 0.006. In 15 dystrophic fibres the size of the available store was 2,807 ± 402 quanta and the probability of release was 0.09 ± 0.012. Neither of these parameters in the dystrophic muscle was significantly different from normal.

Discussion

Choice of experimental procedures and sources of errors

In these experiments, an attempt was made to determine whether or not there were any major abnormalities in the pharmacological behaviour of the neuromuscular junction in murine muscular dystrophy. The

Table 4 Some properties of endplate potentials (e.p.ps) in normal and dystrophic muscle fibres

	Normal	*Dystrophic*
Resting membrane potential (mV)	−16 ± 1.1 (17)	−14 ± 0.9 (16)
Transmitter null potential (mV)	−4.5 ± 0.5 (20)	−4.6 ± 0.4 (20)
Amplitude of e.p.ps at 0.1 Hz (mV)	4.7 ± 1.6 (20)	3.9 ± 1.3 (20)
Rise time (ms)	1.0 ± 0.04 (20)	0.9 ± 0.06 (20)
Time to half decay (ms)	1.7 ± 0.07 (20)	1.5 ± 0.07 (20)
Quantum Contents		
a. 0.1 Hz	167.2 ± 15.5 (17)	261.3 ± 66.3 (17)
b. 1 Hz	135.8 ± 14.6 (17)	198.0 ± 48.2 (17)
c. 10 Hz	126.4 ± 15.4 (17)	192.9 ± 37.3 (17)
d. 30 Hz	98.0 ± 13.7 (17)	148.2 ± 30.4 (17)
e. 100 Hz	91.7 ± 13.2 (17)	136.1 ± 57.2 (17)
Size of the immediately available store	2592.9 ± 324.6 (14)	2806.7 ± 402.4 (15)
Probability of release	0.08 ± 0.01 (14)	0.09 ± 0.01 (15)

Figures are mean ± s.e.mean and the numbers of fibres examined are given in parentheses. The experiments were done on 'cut' muscle fibres. The resting potentials of the cut fibres were set to a standard of −20 mV by passing current through a second microelectrode before e.p.ps were recorded.

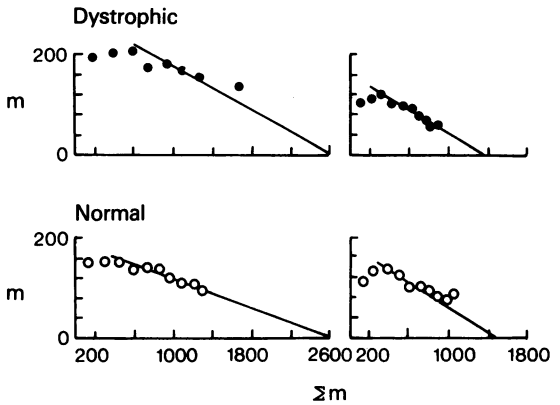

Figure 4 The relationship between the quantum content of successive endplate potentials (e.p.ps, m) and the sum of all preceding e.p.ps (Σm) in 2 normal and 2 dystrophic muscle fibres. The fibres were cut, and the e.p.ps are the first few generated in response to indirect stimulation at 100 Hz. The intercept on the Σm axis (i.e. where m = 0) is taken to indicate the store of available transmitter (n), and the ratio m (of 1st e.p.p.)/n is the probability of transmitter release.

isolated diaphragm was chosen for the experiments even though it is commonly suggested that it is 'spared' the dystrophic process. It should be emphasized, therefore, that the diaphragm was shown to share many of the properties that characterize dystrophic skeletal muscle, in terms of morphological appearance, wet weight and the ability to generate tension. In other respects, the diaphragm seems to be atypical in its involvement in this disease. Thus, the resting membrane potential, the time constant and the input resistance of the dystrophic fibres did not differ significantly from normal; the hind limb muscles usually demonstrate a reduced resting membrane potential (Harris, 1971) and changes in both time constant and input resistance (McComas & Mossawy, 1966; Law & Atwood, 1972).

One of the problems routinely encountered in work on the pathophysiology of diseased tissues is that of systemic errors caused by the biased sampling of 'normal' or 'abnormal' cells or regions in a larger piece of tissue. In the case of our own experiments on dystrophic muscle, microelectrode techniques were used to sample on a random basis a relatively small number of muscle fibres in a given normal or dystrophic piece of muscle. It is difficult to refute the possibility that in the dystrophic muscles such techniques are likely to lead to the biased sampling of larger (and hypothetically healthier) muscle fibres. Our results, showing that the variation of input resistance of dystrophic muscle fibres was no greater than normal (see Table 2) in spite of the larger variation in fibre diameter that characterizes murine muscular dystrophy, may indicate that we had sampled a rather more homogeneous population of dystrophic fibres than we might have expected. However, large muscle fibres, as well as small muscle fibres in dystrophic muscles exhibit all the morphological features of the disease, and Harris & Ribchester (1978) using intracellular dye injection and micro-dissection, have shown unequivocally that the random penetration of fibres in dystrophic muscles does allow the sampling of grossly abnormal muscle fibres. Further, it should be noted that this disease is genetically determined with an autosomal-recessive mode of inheritance. This means that all cell nuclei have to be homozygous for the dystrophic gene. If that gene is fully expressed, as it appears to be in skeletal muscle, the argument that non-dystrophic muscle fibres can exist in a dystrophic muscle is not strong.

Another possible source of error lies in our choice of the cut fibre preparation of Barstad (1962) for the analysis of evoked transmitter release. The preparation was chosen because it is the only preparation available that allows a statistical examination of transmitter release to be made in the absence of transmission-blocking agents. Some sources of error, commonly ignored, such as the correct choice of a value for transmitter null potential and the progressive fall in resting membrane potential, were controlled. Other sources of error are that it is necessary to assume that cutting the muscle fibres has no effect on presynaptic function, and that Poisson statistics are an accurate statistical description of the release process. These assumptions may not be strictly valid for normal systems, and their validity with respect to the dystrophic muscle is virtually untestable.

Experimental results

Contrary to earlier reports (Baker & Sabawala, 1963; Beaulnes *et al.*, 1966), we could detect no differences between normal and dystrophic preparations with regard to sensitivity to (+)-tubocurarine. This discrepancy might be more apparent than real. The experiments reported by Baker & Sabawala (1963) were on isolated diaphragm and peroneus longus muscle, and only in the latter muscles was any significant difference in sensitivity to (+)-tubocurarine established. The experiments by Beaulnes *et al.* (1966) involved measuring the time to transmission block in diaphragm muscles *in vitro* after exposure to a fixed concentration of drug. This technique is so different from that used by Baker & Sabawala and ourselves that it is difficult to compare our respective findings. It seems possible that there are slight differences in the sensitivity of normal and dystrophic preparations to (+)-tubocurarine that are exposed by varying the conditions of an experiment, or that the difference

is seen in only a few muscles. In either case, it seems unlikely that any large and systemic differences exist.

In other experiments the effect of Mg^{2+} on the twitch response of the isolated diaphragm was measured. No differences between normal and dystrophic muscles were noted. Assuming that Mg^{2+} specifically inhibits transmitter release from the motor nerve terminal (del Castillo & Engbaek, 1954), it seems reasonable to draw the inference that transmitter output per impulse (and safety factor for neuromuscular transmission) in dystrophic muscles is broadly similar to normal.

Experiments such as those described above reflect only the responses of a large number of individual muscle fibres; they give no indication of the behaviour of individual junctions. Thus, the gross sensitivity to (+)-tubocurarine and to Mg^{2+} might remain normal when a reduction in nerve-impulse mediated transmitter release is compensated for by an increase in muscle fibre input resistance. Similarly, a change in quantum size might be compensated for by an increase in quantum content, leading to a normal safely factor for neuromuscular transmission and normal sensitivity to agents such as (+)-tubocurarine and Mg^{2+}. The electrophysiological analysis of transmitter release provided one way of examining the behaviour of single neuromuscular junctions.

The mean amplitude and time course of m.e.p.ps in the dystrophic muscle fibres were similar to normal, and it would seem, therefore, that the post-junctional sensitivity to the released transmitter does not differ significantly from normal.

The amplitude and time course of e.p.ps in the dystrophic fibres were similar to normal, and the probability of release, the size of the available store of transmitter and the ability of the nerve terminal to respond to abnormally lengthy periods of stimulation at normal frequencies (the normal rate of discharge in the mouse phrenic nerve is 10 to 12 impulses at approximately 30 Hz per respiratory cycle, Purves & Sackman, 1974) were also indistinguishable from normal.

The quantum content of e.p.ps generated in response to nerve stimulation at all rates ranging from 0.1 to 100 Hz was greater than normal in dystrophic muscle fibres, but the increase was not statistically significant at the 5% level. Of some interest was the observation that the variance of the estimates of quantum content in the dystrophic preparations was greater than normal. One practical consequence of this was that Welch's test (Welch, 1937) had to be used to calculate the significance of the difference between the means. A second was that the sensitivity of the analysis was impaired. As an example of the lack of sensitivity introduced, it can be shown by 'backtracking' through Welch's form of analysis that at 0.1 Hz, for example, a difference between mean quantum contents would only be demonstrated to be statistically significant at the 5% level if the means differed by 1.8 to 2.0 times.

The reason for this very large difference in variance is not clear. It may reflect the true situation in dystrophic fibres. It may also reflect some of the uncertainties inherent in the use of the variance method. Inspection of the data presented in Table 4 shows that the difference between the variance of normal and dystrophic preparations tends to decline as the frequency of stimulation increases. For example, at 0.1 Hz the variance ratio (dystrophic:normal) is approximately 18, and at 30 Hz the ratio is approximately 5. Moreover, this change in variance ratio is primarily due to a relatively large fall in variance in the dystrophic preparations with increasing stimulus frequency. One interpretation of this observation would be that at low rates of stimulation, when large numbers of quanta are being released per impulse, the statistical basis of transmitter release tends towards binomial rather than Poisson and that this tendency is stronger in dystrophic fibres than in normal fibres. Since the cut fibre preparation does not allow one to make a direct measurement of quantum size, this possibility cannot be readily tested, but analysis in terms of Poisson rather than binomial statistics would result in an overestimate of quantum content, and this overestimate may be larger in dystrophic than normal fibres. The possibility that the differences in evoked transmitter release are more apparent than real might also be strengthened by the observation that intact dystrophic diaphragm preparations do not show any marked insensitivity to either Mg^{2+} or (+)-tubocurarine.

In summary, the expression of murine muscular dystrophy does not appear to be complicated by any gross abnormality in the behaviour of the neuromuscular junction. There is no evidence that transmitter release is impaired, even at high rates of stimulation, and so the neuromuscular junction is probably not involved in the development of 'clinical' weakness. It is possible that at low rates of stimulation, transmitter release is greater in dystrophic muscles than normal. Since the safety factor for transmission in mammalian systems is commonly of the order 8 to 10 (Waud & Waud, 1975; Kelly, 1978), an increase in transmitter release would be of little practical consequence to the animal.

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