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EFFECTS OF MUSCLE STRETCH ON TRANSMITTER RELEASE AT END-PLATES OF RAT DIAPHRAGM AND FROG SARTORIUS MUSCLE

By S. A. TURKANIS*

From the Department of Biophysics, University College London, Gower Street, London, WC 1 E 6BT, and the Department of Pharmacology, University of Utah College of Medicine, Salt Lake City, Utah 84112, U.S.A.

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SUMMARY

1. Miniature end-plate potentials (m.e.p.p.s) and end-plate potentials were recorded intracellularly at neuromuscular junctions of the frog sartorius muscle and the rat diaphragm before, during and after muscle stretch.

2. Stretch increased the frequency of the m.e.p.p.s and the mean quantum content (m) at end-plates of the sartorius muscle.

3. Stretch enhanced m.e.p.p. frequency of the sartorius muscle in normal Ringer solution as well as in solutions high in magnesium and low in calcium content. In addition, an increase in frequency occurred in the presence of tetrodotoxin and in the absence of neostigmine methylsulphate.

4. The magnitude of the increase in m.e.p.p. frequency in the sartorius muscle was negatively correlated with the control frequency.

5. In contrast to the above findings at sartorius end-plates, muscle stretch did not affect the frequency of m.e.p.p.s or the m values at end-plates of the rat diaphragm.

INTRODUCTION

One of the many factors that influence transmitter release at frog neuromuscular junctions is skeletal muscle length. Fatt & Katz (1952) found that stretching a frog muscle caused an increase in the frequency of the miniature end-plate potentials (m.e.p.p.s). Hutter & Trautwein in 1956 also examined stretch-induced enhancement of neuromuscular transmission in the frog; they reported that muscle stretch raised both the

* Present address: Pharmacology Department, 2C–202 Medical Center, University of Utah, Salt Lake City, Utah, 84112, U.S.A.

frequency of m.e.p.p.s and the mean quantum content. In the present study, the effects of muscle stretch were investigated on both rat and frog preparations. The findings of Fatt & Katz (1952) and Hutter & Trautwein (1956) on end-plates of the frog were, in general, confirmed. In contrast to the results obtained with frog, stretch had little or no effect on neuromuscular transmission in the diaphragm of the rat. In addition, an attempt was made to elucidate the underlying mechanism of the effects of stretch on frog end-plates.

METHODS

The sartorius muscle and attached motor nerve were excised from the English frog *Rana temporaria* and the American frog *R. pipiens*. In some experiments in which the frequency of miniature end-plate potentials (m.e.p.p.s) was studied, the preparations were bathed in an amphibian Ringer solution of the following composition (mM): NaCl 115·0; KCl 2·0; $CaCl_2$ 1·8. Neostigmine methylsulphate Roche Laboratories) 10^{-6} g/ml. was included in the solution to increase the amplitude of the m.e.p.p.s. In other experiments the Ringer solution was modified: tetrodotoxin (Sigma) or ethylene glycol bis-(2-aminoethyl) tetra-acetic acid (EGTA) (Matheson, Coleman & Bell) was added, or neostigmine methylsulphate was omitted, or the concentrations of magnesium and calcium were altered (see Results).

The diaphragm and phrenic nerve were dissected under ether anaesthesia from Wistar rats weighing 150–250 g. In studies of m.e.p.p. frequency, the diaphragms were continuously bathed in mammalian Ringer solution bubbled with 95% O_2–5% CO_2 (Liley, 1956). Temperatures of the bathing solutions were maintained within the values indicated in the Results by means of a Peltier device (Katz & Miledi, 1963).

Intracellular recording of end-plate potentials (e.p.p.s.) and m.e.p.p.s was made with glass micro-electrodes filled with 3 M-KCl solution. When mean quantum content (m) of the e.p.p. was investigated, rat and frog preparations were bathed in Ringer solutions containing a low concentration of calcium and a high concentration of magnesium to decrease the amount of transmitter released by stimulation of the motor nerve. The motor nerve was placed in a moist chamber, and stimuli were applied through a pair of platinum electrodes. The e.p.p.s were evoked by stimulating the nerve at intervals of 3 sec in the frog and 10 sec in the rat. The m value was calculated from the number of failures and trials (del Castillo & Katz, 1954); the total number of trials ranged from 200 to 250. Mean frequency of the m.e.p.p.s was calculated from the time required for the occurrence of seventy-five to 300 spontaneous potentials.

The preparations were mounted in a specially designed chamber in which the muscle length could be reproducibly altered by means of a modified Narishige A-2 micro-manipulator. Both ends of the muscle were pulled simultaneously in opposite directions by the micro-manipulator. Before muscle length was changed, the micro-electrode was withdrawn from the fibre to prevent damage to the cell. Re-entry into an identified fibre was assured by using surface fibres and making sketches of the area observed with a compound microscope.

The amount of stretch was assessed by counting the number of sarcomeres in about a 50 μm length of muscle fibre adjacent to the end-plate under test. Measurements of m.e.p.p. frequency or m were made successively at a control sarcomere length, at increased length, and again at the control length, using the average m or m.e.p.p. frequency before and after stretch as controls. In frog the initial sarco-

mere length approximated the resting muscle length *in situ*; in rat the control value was obtained by decreasing the tension on the resting muscle until the sarcomere length of the test fibre was minimal. If all three measurements of m.e.p.p. frequency or m were not obtained or the membrane potential decreased more than 10 mV, the experiments were discarded, unless otherwise stated in the Results.

In the present study only findings from surface fibres located near the centre of the sartorius muscle are reported because preliminary results obtained from muscle fibres at the edge of the preparation were inconsistent. In some edge fibres the frequency of m.e.p.p.s increased markedly with stretch; in many others stretch had little or no effect. In contrast, when surface muscle fibres located near the centre of the preparation were stretched, the frequency was usually augmented. The reason for the difference in results from edge and centre fibres is yet unknown.

RESULTS

Effects of stretch on m.e.p.p. frequency. The ratio of m.e.p.p. frequencies (stretch/control) will be referred to as F_f. Only end-plates that showed a stable mean frequency for 20–30 min during the initial control period were used. Nevertheless, the frequencies sometimes differed during the initial and final control periods, in some fibres by as much as 1·5–2·0-fold. In thirteen out of fifteen fibres stretch evoked an increase in frequency. In one end-plate there was a slight decrease; in another there was no change. The mean and standard deviation of the F_f values, in fifteen end-plates from *R. temporaria*, were $2{\cdot}10 \pm 0{\cdot}80$, with a range of 0·95–3·88 (Table 1).

TABLE 1. Effect of skeletal muscle stretch on the frequency of miniature end-plate potentials*

Preparation and bath temperature (°C)	No. of muscle fibres	Frequency (sec^{-1})			F_f†	S.L. during‡ / S.L. before
		Before stretch	During stretch	After stretch		
Frog sartorius§ (19–22)	15	$0{\cdot}89 \pm 0{\cdot}41$	$1{\cdot}79 \pm 0{\cdot}97$	$0{\cdot}91 \pm 0{\cdot}38$	$2{\cdot}10 \pm 0{\cdot}80$	$1{\cdot}22 \pm 0{\cdot}07$
Rat diaphragm (20–22)	27	$1{\cdot}85 \pm 0{\cdot}68$	$1{\cdot}93 \pm 0{\cdot}69$	$1{\cdot}99 \pm 0{\cdot}76$	$1{\cdot}01 \pm 0{\cdot}13$	$1{\cdot}41 \pm 0{\cdot}07$
Rat diaphragm (34–35)	12	$1{\cdot}22 \pm 0{\cdot}32$	$1{\cdot}15 \pm 0{\cdot}45$	$1{\cdot}06 \pm 0{\cdot}45$	$1{\cdot}00 \pm 0{\cdot}20$	$1{\cdot}42 \pm 0{\cdot}07$

* Data represent means and standard deviations of individual values obtained from each muscle fibre studied.

† F_f is the ratio of the frequency of m.e.p.p.s during stretch to the control frequency. The control value is the mean of the frequencies before and after stretch.

‡ S.L., sarcomere length. Ratio of S.L. during stretch to S.L. before stretch; control S.L. in the frog ranged from 2·05 to 2·71 μm and in the rat from 2·07 to 3·23 μm.

§ *Rana temporaria.*

Since the lengths of the sarcomeres adjacent to the end-plate under study were measured, it is certain that all of the fibres were effectively

stretched (Table 1). However, there appeared to be no consistent quantitative relation between the amount of stretch and the enhancement of frequency in different muscle fibres. For example, in one muscle fibre a 1·22-fold increase in sarcomere length raised the m.e.p.p. frequency 2·53-fold; in another fibre a 1·21-fold increase in sarcomere length produced no change in frequency. This variability was even more clearly shown in

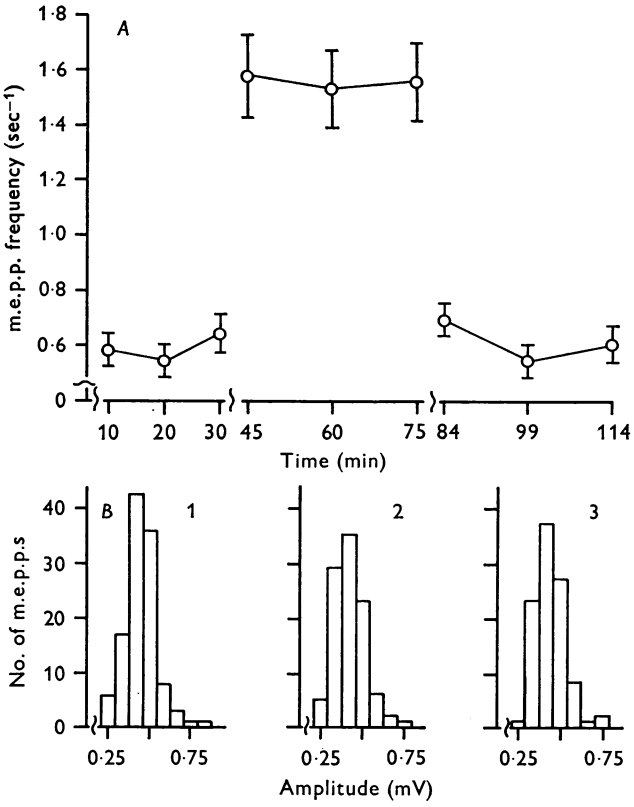

Fig. 1. The effects of muscle stretch on the frequency of m.e.p.p.s in a sartorius muscle fibre of *R. temporaria*. *A*, each circle with brackets represents mean and S.E. of mean of frequency calculated from time required for occurrence of more than 100 m.e.p.p.s. At 10, 20 and 30 min: before stretch, sarcomere length 2·59 μm, average membrane potential 96 mV; at 45, 60 and 75 min: during stretch, 3·17 μm, 89 mV; at 84, 99 and 114 min: after stretch, 2·59 μm, 90 mV. Data illustrate a 2·53-fold increase in frequency of m.e.p.p.s with a 1·22-fold increase in sarcomere length. Broken line on time scale after 30 and 75 min represents time required to complete frequency measurement, to withdraw micro-electrode from fibre, to alter sarcomere length and to re-insert micro-electrode. *B*, amplitude histograms of m.e.p.p.s in *A*. 1, 30 min; 2, 45 min; 3, 84 min. Amplitudes of m.e.p.p.s adjusted for a membrane potential of 96 mV (Katz & Thesleff, 1957). Bath temperature 19° C.

experiments on *R. pipiens* in which the amount of stretch was kept constant in every test.

The time course of the development of the effect could not be followed, as the necessary manipulations between measurements took up some 2–5 min. A representative experiment is depicted in Fig. 1. In the first test period after stretch, enhancement of frequency was present. The increase persisted as long as stretch was maintained and was reversible upon relaxation of the muscle fibre. Fig. 2 also illustrates an augmentation of

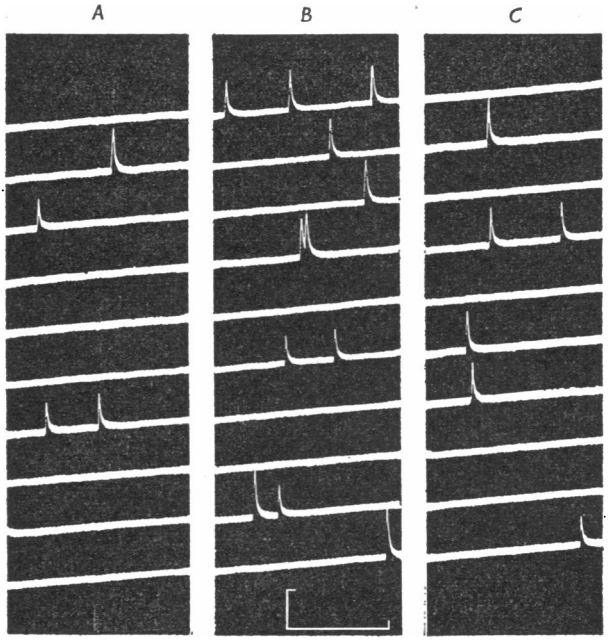

Fig. 2. Effects of stretch on the frequency of m.e.p.p.s in a sartorius muscle fibre of *R. temporaria*. *A*, before stretch, frequency 0·47 sec^{-1}, sarcomere length 2·37 μm, average resting potential 88 mV; *B*, during stretch, 1·33 sec^{-1}, 3·00 μm, 91 mV; *C*, after stretch, 0·67 sec^{-1}, 2·37 μm, 92 mV. Increasing the sarcomere length 1·27 fold increased the frequency 2·33-fold. Consecutive sweeps selected from records of about 100 m.e.p.p.s. Upstrokes retouched for photographic purposes. Calibration, 0·5 sec, 1·0 mV. Bath temperature 20 °C.

frequency caused by stretch. In two muscle fibres the frequency was measured at three different sarcomere lengths and tended to be directly related to the sarcomere length.

The results from six different end-plates were analysed by the χ-square test to check whether the intervals between individual m.e.p.p.s conformed

to a Poisson distribution (cf. Gage & Hubbard, 1965). This was found to be the case, both in the stretch and unstretched condition.

In general, these results agree with those of Hutter & Trautwein (1956) on the extensor digiti IV (toe) muscle of *R. pipiens*. In contrast to the results of their studies, stretch did not always increase the m.e.p.p. frequency in the present investigation. The failure of stretch to augment

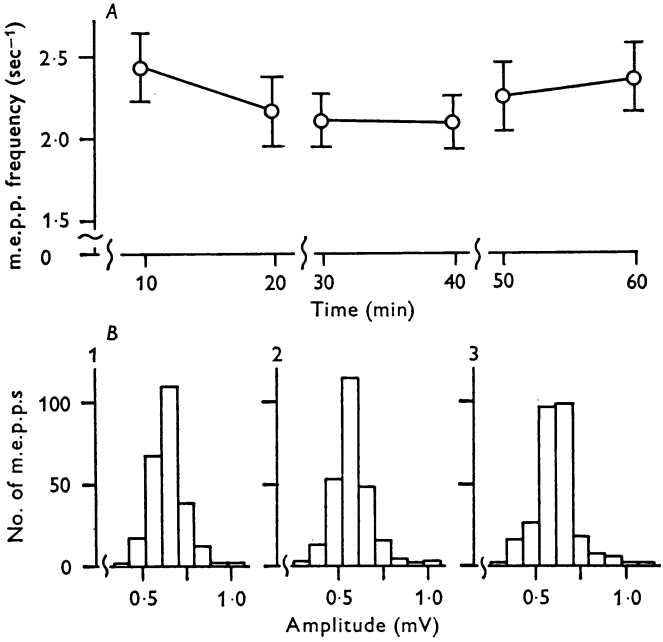

Fig. 3. The effects of muscle stretch on the frequency of m.e.p.p.s of a rat diaphragm fibre. Bath temperature 35° C. *A*, each circle with brackets represents mean and S.E. of mean of frequency calculated from time required for occurrence of more than 100 m.e.p.p.s. At 10 and 20 min: before stretch, sarcomere length 2·41 μm, average resting potential 74 mV; at 30 and 40 min: during stretch, 3·45 μm, 71 mV; at 50 and 60 min: after stretch, 2·41 μm, 66 mV. An increase in sarcomere length (1·43 times control) had little or no effect on frequency. Broken line on time scale after 20 and 40 min, see Fig. 1. *B*, amplitude histograms of m.e.p.p.s in *A*. 1, 10 and 20 min; 2, 30 and 40 min; 3, 50 and 60 min. Amplitudes of m.e.p.p.s adjusted to a membrane potential of 74 mV (Katz & Thesleff, 1957).

m.e.p.p. frequency in the two fibres mentioned above may be explained by a relatively high control m.e.p.p. frequency because in subsequent experiments the size of the enhancement was negatively correlated with the control frequency (see Fig. 5). In additional experiments of six toe muscles (twelve muscle fibres), the observations of Hutter & Trautwein were also

confirmed; in these experiments stretch increased the m.e.p.p. frequency without exception in all fibres tested.

Similar experiments were made to determine the effects of stretch on the frequency of m.e.p.p.s at end-plates in rat diaphragms. In contrast to the results obtained with frog muscle, stretch had no effect on the frequency in rat diaphragm in spite of the fact that the diaphragm fibres were stretched even more than the sartorius fibres (Figs. 3 and 4, Table 1). The mean and S.D. of the F_t values obtained from twenty-seven fibres at 20–22° C were $1{\cdot}01 \pm 0{\cdot}13$ (Table 1); the ratios ranged from 0·81 to 1·20. The F_t was greater than 1·00 in eleven diaphragm fibres and less than 1·00 in sixteen diaphragm fibres. The results indicate that stretch had no effect on frequency on m.e.p.p.s in the rat muscle. In addition, frequency was measured over a wide range of sarcomere lengths in rat diaphragm fibres. A progressive lengthening in sarcomere length from control up to about a 1·5-fold increase did not alter the m.e.p.p. frequency.

The above-mentioned experiments were all made at 20–22° C. In addition, twelve diaphragm fibres were studied at higher temperatures. As indicated in Table 1, raising the bath temperature had no effect on the results. The mean and S.D. of the F_t values for twelve diaphragm fibres at 34–35° C were $1{\cdot}00 \pm 0{\cdot}20$.

Because the usual experimental procedures required an interval of 2–5 min between controls and experimental measurements, it seemed possible that a transient augmentation of frequency may have been missed. At four rat end-plates, the frequency measurements were begun within 1 min after stretch and the results again showed no increase in frequency.

Modification of Ringer solution on stretch-induced increase in m.e.p.p. frequency. The following experiments were made in an attempt to elucidate the mechanism of the stretch-induced increase in m.e.p.p. frequency in the frog. To reduce variability certain standard procedures were adopted. A constant amount of stretch, increasing the sarcomere length to 1·2 times the control, was used in each fibre. Surface end-plates were selected from the central portion in the tibial part of the muscle. In addition, all the experiments were carried out on a single batch of *R. pipiens* with the bath temperature maintained between 18 and 19° C. The results from at least two muscles were pooled and at least five fibres were investigated in each preparation. As controls, twenty-two muscle fibres were examined in normal frog Ringer solution containing neostigmine methylsulphate (see Methods). The mean and S.D. of the F_t values of the controls were $2{\cdot}44 \pm 0{\cdot}38$ with a range from 1·03 to 5·10.

Fourteen end-plates were studied in a modified Ringer solution containing 10 mM magnesium and 0·10 mM calcium. As indicated in Table 2, the mean and S.D. of the F_t values were $2{\cdot}35 \pm 0{\cdot}94$ with a range from 1·34

to 4·65. The results were similar to those obtained with normal Ringer solution. Thus, a change in the magnesium to calcium ratio does not appear to affect the increase in frequency; such findings are again in agreement with those reported by Hutter & Trautwein (1956).

TABLE 2. Effects of agents on the stretch-induced increase in frequency of the miniature end-plate potentials in frog muscle*†

Ringer solution containing:	No. of fibres	F_t‡	F_{tc}§
Neostigmine methylsulphate‖ (10^{-6} g/ml.)	22	2·44 ± 0·38	2·39 ± 0·68
Neostigmine methylsulphate and TTX (10^{-6} g/ml.)	17	1·84 ± 0·49	1·94 ± 0·70
Neostigmine methylsulphate and Ca (0·1 mM) and Mg (10·0 mM)	14	2·35 ± 0·94	2·49 ± 0·71
No drug	11	2·64 ± 0·72	2·40 ± 0·70

* Sartorius muscle fibres from *R. pipiens*.

† Data represent means and S.D. of individual values obtained from each muscle fibre studied.

‡ F_t, as in Table 1.

§ F_{tc} is F_t corrected for differences in control frequencies by analysis of covariance. F = variate; log of control = covariate.

‖ Normal Ringer solution; control experiments.

The muscles were bathed in the Ringer solution for 1 hr before measurements were taken. Muscle fibres were stretched 1·2 times the control sarcomere length, which ranged from 2·27 to 2·55 μm.

Neostigmine methylsulphate treatment, four muscles; tetrodotoxin (TTX) treatment, three; others, two.

When solutions were used containing 1·0 mM-EGTA and 7·0–20·0 mM magnesium, the frequency and amplitude of the m.e.p.p.s before stretch were markedly reduced; similar results have been reported by Miledi & Thies (1971). Furthermore, the muscles were very easily damaged by stretch, as indicated by a sharp decrease in the membrane potential and visible signs of fibre damage. Therefore, it was not possible to measure the frequency before, during and after stretch in seven different preparations. However, in one preparation bathed in a modified Ringer solution containing 1·0 mM-EGTA and 7·0 mM magnesium for 1 hr, the frequency was successfully measured before and during stretch in three muscle fibres. In spite of the fact that control measurements after stretch were unobtainable, the ratios of the frequencies during stretch to those before stretch were calculated to be 3·50, 1·17, and 1·93; similar ratios were obtained with normal Ringer solution. It appears that the augmentation of m.e.p.p. frequency can occur in very low concentrations of calcium.

To test whether the presence of neostigmine had anything to do with the

observed effect, eleven muscle fibres from the sartorius muscle of the frog were investigated in a Ringer solution without anticholinesterase. As indicated in Table 2, the mean and standard deviation of F_t values were $2{\cdot}64 \pm 0{\cdot}72$; the individual values ranged from 1·97 to 4·00. The results suggest that the use of anticholinesterase was not related to the observed increase in m.e.p.p. frequency.

Seventeen sartorius fibres were examined in Ringer solution containing tetrodotoxin (10^{-6} g/ml.). The mean and S.D. of the F_t values were $1{\cdot}84 \pm 0{\cdot}49$ (Table 2); the individual values ranged from 0·97 to 2·64. Thus, stretch

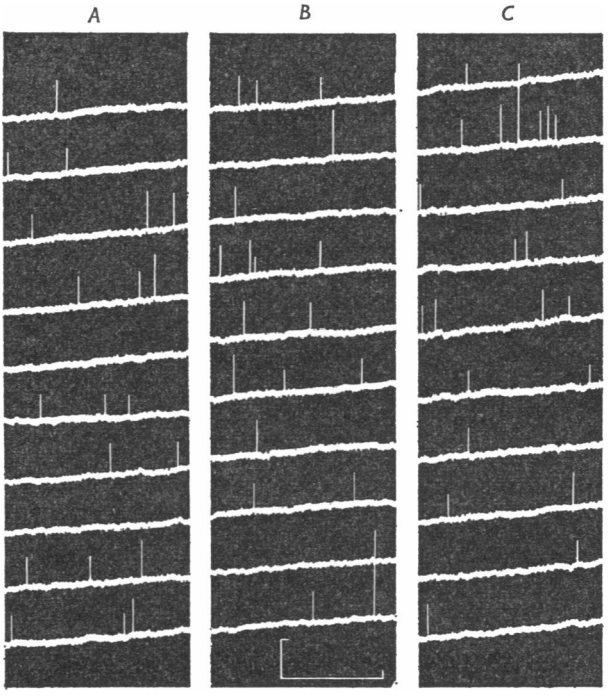

Fig. 4. Effects of stretch on the frequency of m.e.p.p.s of a rat diaphragm fibre. *A*, before stretch frequency 2·29 sec^{-1}, sarcomere length 2·41 μm, average resting potential 74 mV; *B*, during stretch 2·09 sec^{-1}, 3·45 μm, 71 mV; *C*, after stretch, 2·30 sec^{-1}, 2·41 μm, 66 mV. An increase in sarcomere length (1·43 times control) had little or no effect on frequency. Consecutive sweeps selected from records of about 100 m.e.p.p.s. Upstrokes retouched for photographic purposes. Calibration, 0·5 sec, 1·0 mV. Bath temperature 35° C.

evoked an enhancement of the frequency in tetrodotoxin. Although the mean, S.D. and range are slightly lower, the experimental results with tetrodotoxin are not significantly different from those with normal Ringer solution (see below).

It appeared that there was some relation between size of the increase in frequency caused by stretch and the control frequency (Fig. 5). There was a significant negative correlation between the F_t values and the log of the control frequencies ($P < 0{\cdot}005$). Since the F_t values were related to the log of the control frequencies, the analysis of covariance was utilized to correct for differences in the control frequencies (Snedecor & Cochran,

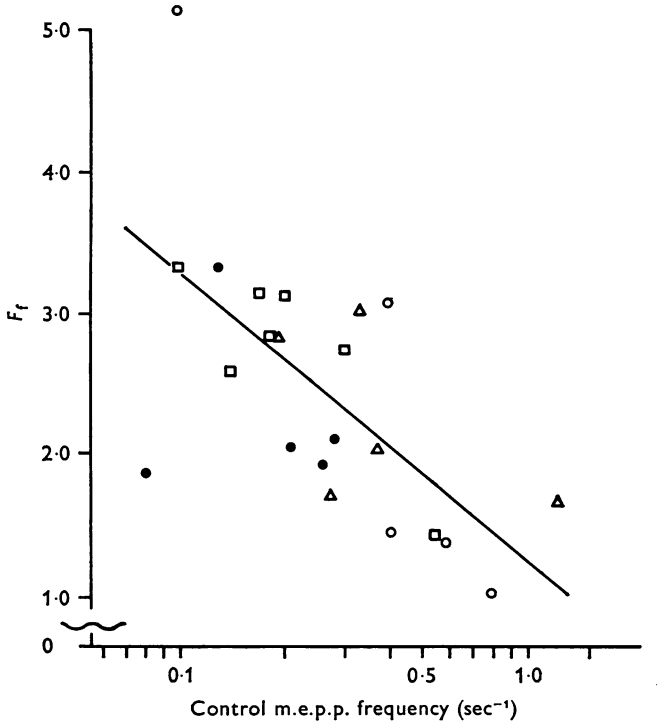

Fig. 5. Relation between F_t and control m.e.p.p. frequency. F_t is the ratio of frequency during stretch to control frequency. Control m.e.p.p. frequency is mean of the frequencies before and after stretch. Each point indicates results from one end-plate; each symbol represents a different muscle bathed in normal Ringer solution. Continuous line shows the logarithmic linear relation; correlation coefficient is $-0{\cdot}64$. Sarcomere stretched 1·2 times control length in each muscle fibre. Bath temperature 18–19° C.

1967). Data from the above-described experiments involving modifications of the Ringer solution (low calcium and high magnesium, no neostigmine, tetrodotoxin) were subjected to analysis of covariance. The F_t values were the variates; the log of the control frequencies were the covariates. Means and S.D. of F_t values corrected for differences in control frequency are listed in Table 2 under F_{tc}. The results of the analysis of covariance suggest that modifying the Ringer solution did not significantly

alter the results ($P > 0{\cdot}10$). Thus, even when using this correlation to correct for differences in control frequencies, no further change became apparent.

Effects of stretch on m. The effect of stretch on the mean quantum content of the end-plate potentials (m) was investigated both in the sartorius muscle (*R. temporaria*) and the diaphragm of the rat (Table 3). The ratio of m values (stretch/control) will be referred to as F_m. The mean and S.D. of F_m values calculated from data obtained from ten sartorius muscle fibres were $2{\cdot}69 \pm 1{\cdot}4$ with a range of 1·11 to 5·0. In the test fibres the mean sarcomere length was increased $1{\cdot}24 \pm 0{\cdot}1$ times the control (Table 3). Similar results were reported for the frog toe muscle by Hutter & Trautwein (1956).

TABLE 3. Effects of skeletal muscle stretch on the quantum content (m)*

Preparation and bath temperature (°C)	No. of muscle fibres	Quantum content: Before stretch	During stretch	After stretch	F_m†	S.L. during‡ / S.L. before
Frog sartorius§ (19–22)	10	$0{\cdot}66 \pm 0{\cdot}41$	$1{\cdot}54 \pm 0{\cdot}74$	$0{\cdot}63 \pm 0{\cdot}44$	$2{\cdot}69 \pm 1{\cdot}4$	$1{\cdot}24 \pm 0{\cdot}01$
Rat diaphragm (20–22)	12	$0{\cdot}82 \pm 0{\cdot}56$	$0{\cdot}80 \pm 0{\cdot}56$	$0{\cdot}80 \pm 0{\cdot}52$	$0{\cdot}98 \pm 0{\cdot}12$	$1{\cdot}38 \pm 0{\cdot}07$

* Data represent means and S.D. of individual values obtained from each muscle fibre studied.

† F_m is the ratio of m during stretch to control m; the control is the mean of m values before and after stretch.

‡ Ratio of sarcomere length during stretch to sarcomere length before stretch; control sarcomere length in the frog ranged from 2·07 to 2·37 μm; in the rat, 2·10 to 3·36 μm.

§ *R. temporaria.*

In contrast to the frog muscle, stretching the diaphragm of the rat did not increase m (Table 3). The mean and standard deviation of F_m in twelve diaphragm fibres at 20–22° C were $0{\cdot}98 \pm 0{\cdot}12$ with a range of 0·80–1·22. The mean sarcomere length of the diaphragm fibres was increased $1{\cdot}38 \pm 0{\cdot}07$ times the control measurements. In four muscle fibres maintained at 34–35° C, similarly negative results were obtained.

DISCUSSION

The results confirm that muscle stretch increases the frequency of miniature end-plate potentials (m.e.p.p.s) and the mean quantum content of the end-plate potential (m) in sartorius and toe muscles of the frog.

It may be assumed that stretch acts directly on the nerve terminals for changes in the value of m and frequency of m.e.p.p.s are a clear indicator of a presynaptic site of action. In contrast to the findings with these frog preparations, stretch does not affect either m or the frequency of m.e.p.p.s in the rat diaphragm.

The reason for the different results at frog and rat end-plates is a matter for conjecture; however, it may be due to major differences in the morphology and geometrical arrangements of their nerve terminals. Neuromuscular junctions of frog twitch fibres, and in particular their terminal axon branches, are elongated structures about 100–500 μm in length (Katz, 1969; Kuno, Turkanis & Weakly, 1971; R. Miledi, personal communication). In contrast, the nerve terminals of the rat diaphragm are convoluted within a circular disk of 20–30 μm diameter (Cole, 1957). It is possible that frog nerve terminals are susceptible to stretch because of their longitudinal orientation along the muscle fibre, while the terminals of mammalian end-plates may undergo relatively very little deformation when the muscle fibre is lengthened.

Beyond that, the present experiments do not allow one to speculate on the mechanism underlying the stretch effect. The absence of any change resulting from alterations in the external calcium/magnesium ratio makes it very unlikely that the effect can be mediated via depolarization of the axon membrane (see also Hutter & Trautwein, 1956), and some other factor has to be looked for, possibly a structural rearrangement whereby synpatic vesicles are brought into a more effective proximity to the surface membrane.

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