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PHYSIOLOGICAL REGULATION OF SYNAPTIC EFFECTIVENESS AT FROG NEUROMUSCULAR JUNCTIONS

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SUMMARY

1. Nerve terminals in two different muscles of the frog, the sartorius and cutaneous pectoris (c.p.), have been found to differ sharply in safety factor. This difference is shown to be attributable to corresponding disparities in the amount of transmitter released, without evident correlated morphological differences.

2. In Ringer containing 0·3 mM-Ca^{2+} and 1 mM-Mg^{2+}, quantal content of c.p. junctions exceeded that of sartorius junctions by 3·4 times.

3. When quantal content was corrected for nerve terminal size, c.p. terminals still released 2·4 times more transmitter per unit terminal length.

4. Light and electron microscopic examination of junctional morphology in the two muscles revealed no significant difference in the spacing of presynaptic active zones, the width of synaptic contact, or the density of presynaptic vesicles and mitochondria. It seems likely, therefore, that the greater release at c.p. junctions is due to a 'physiological' difference between the two populations of terminals.

5. No evidence could be found that action potential invasion of the terminal was less complete in the sartorius than in the c.p.

6. The dependence of evoked and spontaneous release on Ca^{2+} concentration was of similar slope for terminals in the two muscles, but of different absolute value, consistent with the observed difference in release.

INTRODUCTION

It is widely accepted that every fast twitch muscle fibre of the frog is supplied with at least one neuromuscular junction where the safety factor for transmission is high (Katz & Kuffler, 1941; Fatt & Katz, 1951). However, in the course of studying the interaction between foreign nerve terminals on frog muscle fibres (Grinnell, Rheuben & Letinsky, 1977; Grinnell, Letinsky & Rheuben, 1979), we have observed that normal junctions in the frog sartorius muscle are much more easily blocked by D-tubocurarine chloride or by Ringer solutions containing low Ca^{2+} or high Mg^{2+} concentrations than are normal junctions of the cutaneous pectoris muscle. Indeed, in the species studied (*Rana pipiens* and *R. catesbeiana*), many sartorius fibres are innervated exclusively by junctions that are subthreshold to a single stimulus even

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in normal Ringer solution (Herrera & Grinnell, 1980). This observation confirms that made by Kuffler (1952) for the frog submentalis muscle.

Since little is known of the factors that regulate synaptic size and effectiveness, we have sought explanations for the observed differences in safety factor. Analysis of the structure and release properties of identified junctions in the two muscles reveals that differences in synaptic size and muscle fibre input impedance can account for only a small part of the disparity. Nerve terminals of similar size and appearance in the two muscles can release very different amounts of transmitter. This study suggests that physiological plasticity in transmitter release may play an important role in the normal regulation of synaptic effectiveness. A preliminary report of these results has been published (Herrera & Grinnell, 1980).

METHODS

Sartorius and cutaneous pectoris (c.p.) muscles were dissected from pithed *Rana pipiens* and *R. catesbeiana* at various times of the year. Muscles were pinned into a Sylgard-lined bath (approximately 10 ml. volume) at 1·05 or 1·1 times the rest length measured in the intact animal. For some experiments, muscles were stretched so that the distance between adjacent sarcomeres in the middle of the muscle was 2·4 μm (approximately 1·05–1·1 times normal rest length for both muscles). Sarcomere spacing was observed using Hoffman Modulation-Constrast optics with a Zeiss 40 × 0·75 water immersion objective (final magnification 800 ×). The bath was filled with normal frog Ringer consisting of 116 mM-NaCl, 2 mM-KCl, 1·8 mM-$CaCl_2$, buffered to pH 7·2 with approximately 1 mM-$NaHCO_3$. The Ca^{2+} concentration of 1·8 mM was determined by direct measurement to be the same as that of frog plasma. For most experiments 1 mM-$MgCl_2$ was added to maintain adequate divalent cation concentration when Ca^{2+} concentration was lowered. Solutions were changed by perfusing the bath with at least 10 times its volume. The drug D-tubocurarine chloride (dTC, Nutritional Biochemicals) was dissolved in Ringer at 10^{-4} M to form a stock solution. Bath temperature was maintained at 15 °C within 0·5 °C. Denervations were performed by nerve section after anaesthetizing frogs in 0·2 % tricaine methanesulphonate (Ayerst).

Safety factor measurements. The safety factor for transmission was estimated by recording isometric twitch tension evoked by nerve stimulation while varying Ca^{2+} concentration or partially blocking with dTC. Twitch tension in Ringer solution with altered Ca^{2+} concentration was expressed as a percentage of the control tension produced in normal Ringer. For the c.p., this percentage was taken to represent the proportion of fibres in the muscle where the end-plate potential was above threshold for generation of a muscle action potential. This is not strictly true for the sartorius, since not all sartorius fibres are active in normal Ringer (see below). Nevertheless, such a measurement does approximate the proportion of active fibres in the sartorius. Thus the extent to which tension is blocked by a given low Ca^{2+} concentration is a measure of the safety factors at junctions in that muscle. Isometric twitch tension was measured with a Statham UC3 transducer or a more sensitive device built in our laboratory. Maximum sensitivity was 1·1 mV/mg. Stimuli were delivered to the nerve at 1 Hz with suction electrodes (3–10 V, 0·1–1 ms duration). Initially tension measurements were made with the muscles stretched to approximately 1·1 (c.p.) and 1·05 (sart.) times rest length. In subsequent intracellular recording, the length was carefully maintained at 1·1 times rest length in the c.p. and either 1·1 or 1·05 times rest length in the sart.

Electrophysiology. Muscle fibres were impaled with glass micropipettes filled with 3 M-KCl (30–50 MΩ). To measure input impedance or action potential threshold, two micro-electrodes, for current injection and potential recording, were inserted into the same fibre at less than 50 μm separation. For measurement of quantal content, muscles were bathed in Ringer containing 0·3 mM-Ca^{2+} and 1 mM-Mg^{2+} to partially block release. End-plate potentials (e.p.p.s) were recorded near synaptic sites while stimulating the nerve at a frequency of 1 Hz. No facilitation was seen at this stimulus frequency. Each synaptic potential was corrected to a standard resting potential of −90 mV and corrected for non-linear summation with the methods of Katz &

Thesleff (1957) and Martin (1955). Miniature e.p.p.s were photographed from the oscilloscope onto moving film while e.p.p.s were either filmed or directly averaged with a Hewlett-Packard 5480A signal analyser. Quantal content was calculated by the direct method ($m = \overline{\text{e.p.p.}}/\overline{\text{m.e.p.p.}}$), although in a few cases the method of failures (m = ln (no. of trials)/(no. of failures)) was also used. These values were generally in good agreement. In most experiments, junctions were marked by intracellular injection of a dye solution from a third micropipette which was bevelled and filled with 4·5% Chicago blue and 0·1 M-K acetate. After passing current to verify that the tip of this electrode was in the correct fibre, a pulse of pressure (0·7–2·8 kg/cm^2) was applied to eject the dye.

Microscopy. The nitroblue tetrazolium (NBT) method (Letinsky & DeCino, 1980) and acetylcholinesterase stain (Karnovsky, 1964) were used to stain the nerve terminal and the muscle end-plate. Muscles stretched to their rest length were prefixed with 2% glutaraldehyde in Ringer for 20 min. After prefix they were transferred directly to NBT staining solution containing 10 mg NBT and 1 mg phenazine methosulphate in 10 ml. Ringer with 2% glutaraldehyde for about 4 min. After a brief wash with Ringer, the muscles were stained for cholinesterase and post-fixed with 2% glutaraldehyde. The intracellularly injected dye marks survived this procedure and were used for identification of those junctions (hereafter termed 'identified junctions') whose physiology had been analysed. After dissecting and orienting each identified fibre to view the junction well, camera lucida drawings of the stained junction were made. Nerve terminal size was measured from these drawings as the summed length of the individual nerve terminal branches. Slight differences in the degree of stretch were compensated by correcting all measurements of nerve terminal length to a standard sarcomere spacing of 2·2 μm.

For electron microscopy, muscles were pinned at rest length, stained for cholinesterase as above, fixed in 2% glutaraldehyde in Ringer, post-fixed in 1% OsO_4, dehydrated in ethanol, rinsed in tuolene, and embedded as a whole mount in a thin wafer of Epon. Measurements were made from electron micrographs at a final magnification of 30,000.

All data are presented as mean ± standard error of the mean.

RESULTS

Differences in safety factor

Differences in safety factor between sartorius (sart.) junctions and cutaneous pectoris (c.p.) junctions were tested by delivering single brief stimuli to the nerve and measuring isometric twitch tension while varying curare concentration. The

TABLE 1. Effect of D-tubocurarine on twitch tension in *Rana pipiens*. Data, which are from a typical experiment, represent twitch tension in curare-containing solution as a percentage of control tension in normal Ringer solution

	10^{-6} M-dTC (%)	$2+10^{-6}$ M-dTC (%)
C.p.	82	31
Sart.	14	1

results of a typical experiment are shown in Table 1. One can see that a given curare concentration produces a more pronounced block of sartorius tension than of c.p. tension. On the assumption that all fibres contribute about equally to tension, the percentage of control tension can be taken to represent the proportion of fibres with e.p.p.s above threshold. It is important to recognize that, for multiply innervated sartorius fibres, block of twitch means that transmitter release at all junctions on the fibre was reduced below threshold.

This difference in safety factor, shown with post-synaptic block, was investigated more thoroughly with presynaptic block. Twitch tensions were measured while

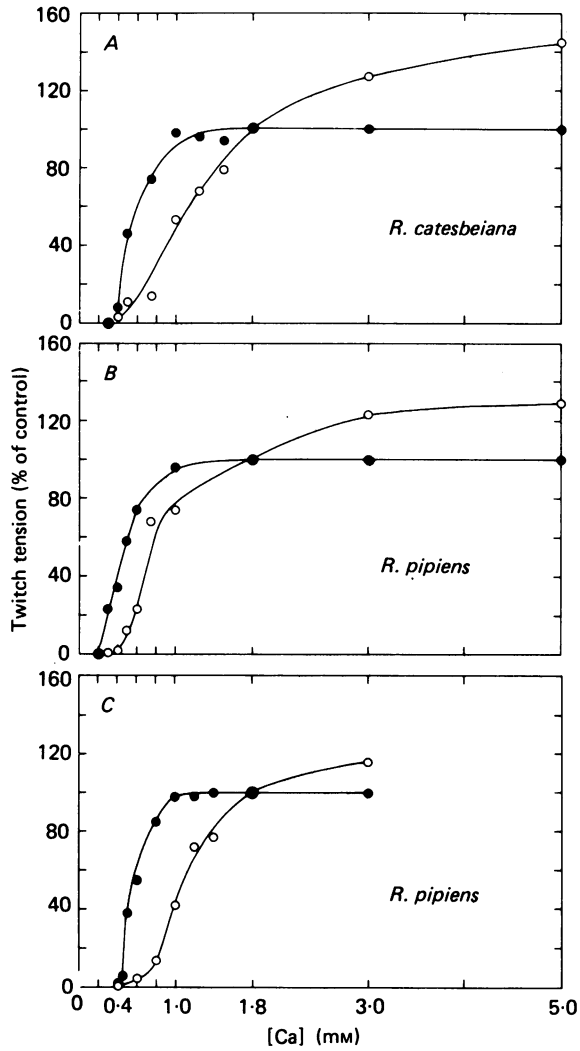

Fig. 1. The effect of Ca^{2+} concentration on isometric twitch tension evoked by nerve stimulation. Tension is expressed as a percentage of the tension generated in normal Ringer containing 1·8 mM-Ca^{2+}. Each point is the mean of two to ten muscles. Curves are drawn by eye. *A*, *B*: Ringer without Mg^{2+}; *C*: Ringer with 1 mM-Mg^{2+}. ●, cutaneous pectoris (c.p.); ○, sartorius.

varying Ca^{2+} concentration in both *R. catesbeiana* and *R. pipiens* preparations (Fig. 1). A decrease in Ca^{2+} concentration from 1·8 to 1·0 mM had only a slight effect on c.p. tension but caused a substantial drop in sartorius tension. As the Ca^{2+} concentration in the Ringer was raised above 1·8 mM, there was a sizeable increase in tension in the sartorius. This strongly suggests that at 1·8 mM-Ca^{2+} concentration, which was verified to be that of normal frog plasma (B. Ehrlich, personal communication), a large number of fibres are innervated exclusively by junctions which release subthreshold quantities of transmitter, and that these junctions were made supra-

threshold by raising the Ca^{2+} concentration. Note that the c.p. does not show increased tension in high Ca^{2+} concentration, suggesting that release from all c.p junctions is suprathreshold in normal Ringer. From the shape of these two curves, it can be concluded that the c.p. junctions form a more uniform population, while the strongest synapse on each sartorius fibre varies in effectiveness over a much wider range. Similar results were obtained if 1 mM-$MgCl_2$ was added to all solutions (Fig. 1*C*), with the expected shift to the right due to the competitive effect of Mg on transmission. In the remainder of the results to be presented, 1 mM-$MgCl_2$ was added to all solutions to maintain adequate divalent cation concentration.

Several possible explanations for this difference is safety factor were investigated: (1) direct effects of changing Ca^{2+} concentration on contraction; (2) differences in muscle action potential threshold; (3) difference in post-synaptic input impedance; (4) differences in presynaptic transmitter release. The results presented below show that the first three possibilities are of little or no relevance and that the difference in safety factor is largely due to differences in presynaptic transmitter release.

Direct effects of Ca on contraction. Control experiments showed that changing Ca^{2+} concentration over the range shown in Fig. 1 had no significant effect on the tension produced by direct supramaximal electrical stimulation of the muscle.

Differences in action potential threshold. Using two micro-electrodes, we measured muscle fibre action potential threshold as a function of Ca^{2+} concentration in Ringer solution containing 1 mM-Mg^{2+}. Table 2 summarizes these data. Two conclusions could be drawn: (1) the dependence of threshold upon Ca^{2+} concentration was the same for the two muscles, i.e. raising Ca^{2+} concentration from 0·5 to 1·8 mM made threshold less negative by about 6 mV without significantly changing resting membrane potential; (2) at any one Ca^{2+} concentration, there was no significant difference in threshold between the two muscles.

TABLE 2. Muscle fibre action potential threshold, expressed as the difference between resting potential and threshold potential, as a function of Ca^{2+} concentration. Number of fibres is in parentheses

	Relative threshold (mV)	
$[Ca^{2+}]$ (mM)	C.p.	Sart.
0·5	20·3 ± 0·9 (6)	20·9 ± 0·7 (12)
1·0	25·2 ± 1·0 (10)	26·8 ± 1·8 (6)
1·8	26·3 ± 1·6 (10)	26·9 ± 0·8 (13)
3·0	30·5 ± 0·7 (10)	33·0 ± 0·5 (10)

Differences in input impedance (R_{in}). Fibres of the c.p., in addition to being shorter than those of the sartorius, have a somewhat smaller mean diameter. Consistent with this is a slightly larger mean R_{in} and miniature end-plate potential (m.e.p.p.) amplitude. Fig. 2 shows that the data from identified fibres in the two muscles fall approximately along the same curve, with considerable overlap, but that sartorius fibres predominate at the large diameter end, c.p. fibres at the small diameter end. For the sartorius fibres studied in this respect, the mean R_{in} was 0·63 ± 0·06 MΩ (S.D., n = 47); for the c.p. fibres, 0·91 ± 0·04 MΩ (S.D., n = 50). This corresponds to a c.p./sartorius ratio of approximately 1·4. Thus there is a population of large sar-

torius fibres with correspondingly small m.e.p.p.s, on which synapses with the same quantal contents as normal c.p. synapses might be subthreshold. This presumably explains part of the difference in safety factor between the two muscles. On the other hand, direct measurements of quantal content (see below) support the idea that the differences in safety factor is due in large part to greater release at c.p. terminals.

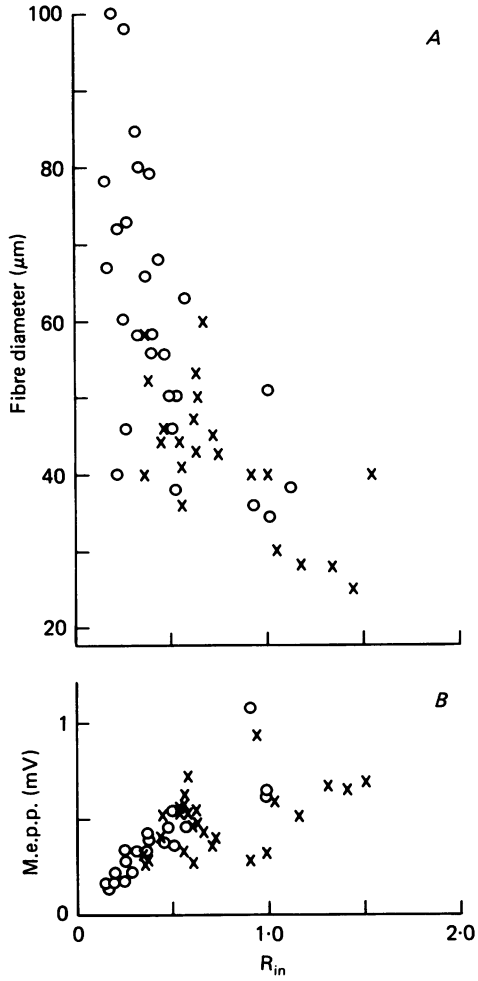

Fig. 2. Muscle fibre diameter (*A*) and m.e.p.p. amplitude (*B*) *vs.* input impedance in identified muscle fibres, i.e. those marked fibres where input impedance, synaptic physiology, and anatomy could be correlated. Fibre diameters were measured in the intact muscle with a light microscope. ×, cutaneous pectoris (c.p.); ○, sartorius.

Differences in transmitter release

Upon investigation with intracellular recording techniques, it became apparent that at a given Ca^{2+}/Mg^{2+} ratio, junctions in the two muscles differed sharply in quantal content (Table 3).

By convention, while measuring twitch tension we had stretched the c.p. muscle to 1·1 times its *in situ* rest length and the sartorius muscle to 1·05 times its rest

length. In our test solution under these conditions, quantal content at c.p. junctions averaged 3·7 times that of sartorius junctions (Table 3). Since the degree of stretch is known to affect transmitter release (Libet, Ralston & Feinstein, 1951; Hutter & Trautwein, 1956; Turkanis, 1973), we compared quantal contents when the sartorius was also stretched to 110%. Even though this further stretch enhanced transmission in the sartorius, c.p. quantal contents still averaged 3·3 times higher under the same conditions (Table 3). In separate experiments, the degree of stretch was monitored

TABLE 3. Quantal content in 0·3 mM-Ca^{2+}, 1 mM-Mg^{2+}

	Quantal content	$m_{c.p.}/m_{sart.}$
1. C.p. at 110% *in situ* length	$6{\cdot}89 \pm 0{\cdot}61$, $n = 69$	—
2. Sart. at 105% *in situ* length	$1{\cdot}89 \pm 0{\cdot}27$, $n = 42$	3·7
3. Sart. at 110% *in situ* length	$2{\cdot}11 \pm 0{\cdot}37$, $n = 24$	3·3

by sarcomere spacing rather than whole muscle length. When both muscles were stretched to a sarcomere spacing of 2·4 μm, c.p. quantal content ($6{\cdot}25 \pm 1{\cdot}97$, $n = 16$) still exceeded sartorius quantal content ($2{\cdot}89 \pm 1{\cdot}10$, $n = 14$) by 2·1 times.

Quantal content at frog neuromusuclar junctions is known to be proportional to nerve terminal size (Kuno, Turkanis & Weakly, 1971; Bennett & Raftos, 1977). Determination of terminal diameter or area of contact is extremely difficult in acetylcholinesterase stained preparations using light microscopy as was the case in these measurements, but no consistent difference in these dimensions was seen between the two muscles. Careful electron microscope analysis of transverse sections of randomly selected terminals from the two muscles reveals no significant difference in terminal diameter or width of synaptic contact (see below), lending support to our working hypothesis that synaptic length is an adequate measure of synaptic size. Moreover, using a new nerve terminal stain developed by Letinsky & DeCino (1980), which is fully compatible with the Karnovsky cholinesterase stain, we ascertained that the nerve terminal does in fact normally correspond accurately, in length, to the associated post-synaptic gutter revealed by the ACh esterase stain. We therefore measured the length of a large number of randomly selected terminals from the two muscles. Presumably this sample is very similar to the random sample for which the 3·3-fold difference in quantal content was determined. Mean nerve terminal length were 335 ± 13 μm, $n = 247$ (c.p.) and 329 ± 11 μm, $n = 282$ (sart.), a ratio of 1·02. Clearly terminal length alone cannot explain the greater c.p. quantal content.

To make this comparison more accurate, and to be able to determine whether the difference in quantal content needed to be explained by an inherent difference in release, we correlated the morphology of a number of identified junctions with their release properties. We first measured quantal content at a given junction in low Ca^{2+}, then marked that junction by intracellular dye injection for identification and measurement of junctional size following histological processing. The results, expressed as the number of quanta released in the test solution per 100 μm of terminal length, are shown in Fig. 3. One can see (compare Fig. 3*A*,*B*) that release per unit terminal length is sensitive to stretch, confirming and extending earlier observations (Hutter & Trautwein, 1956). However, even if the two muscles are stretched to the

same relative extent (compare Fig. 3*B*, *C*), c.p. terminals still average 2·4-fold greater release per unit length. In other muscles stretched to a constant sarcomere spacing (2·4 μm), release per unit length in c.p. junctions (1·16 $\pm$ 0·33 quanta/100 μm, n = 16) exceeded that of sartorius junctions (0.36 $\pm$ 0·16 quanta/100 μm, n = 14) by 3·2 times.

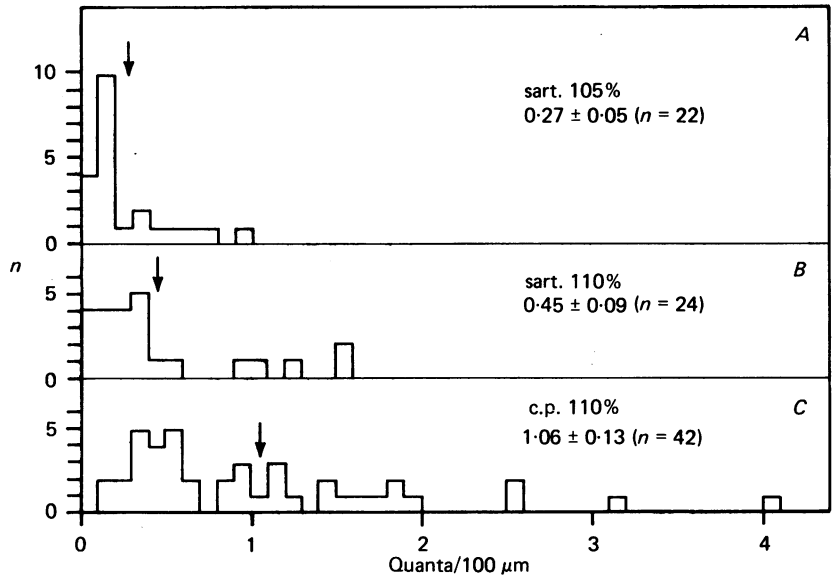

Fig. 3. Quanta released per 100 μm nerve terminal length in Ringer containing 0·3 mM-Ca^{2+} and 1 mM-Mg^{2+}. *A*: sart. at 105% rest length; *B*: sart. at 110% rest length; *C*: c.p. at 110% rest length. Arrows indicate means.

In order to insure that this difference in quantal content was not unique to the situation where the probability of release was lowered by low Ca^{2+} and high Mg^{2+}, we compared e.p.p. size at identified end-plates in curare blocked muscles. With a curare concentration of 3×10^{-6} M, e.p.p. amplitude in the c.p., normalized to nerve terminal length, exceeded that in the sartorius by approximately 1·9-fold, when both were corrected for non-linear summation and input resistance (c.p. 1·5 $\pm$ 0·2 mV/100 μm terminal length, n = 14; sart. 0·8 $\pm$ 0·3 mV/100 μm terminal length, n = 13). Thus the greater release per unit length shown by c.p. terminals is also found at normal Ca^{2+} concentrations.

Possible explanations for the difference in release

Morphological comparisons of c.p. and sartorius terminals. We next investigated whether there was an evident difference in presynaptic morphology that could explain the greater release from c.p. terminals. It can be postulated that, for any given length of nerve terminal, the amount of transmitter released could depend on the number of active zones, the area over which release occurs (i.e. the width of synaptic contact at active zones), and the number of packets of transmitter available for release. Release may also depend on the number or the Ca^{2+}-sequestering activity of mito-

chondria in the nerve terminal (Alnaes & Rahamimoff, 1975). There are, of course, many other physiological processes involved in release, any one of which could be important in this regard; but in our search for a morphological correlate, we examined (1) the spacing of active zones, (2) the width of synaptic contact, (3) the number of presynaptic vesicles (possibly a reflexion of the number of packets of transmitter available), and (4) the number of mitochondria in cross-sections of terminals from the two muscles. Pl. 1 shows how these comparisons were made.

In order to compare the longitudinal spacing of presynaptic active zones, we took advantage of the observation that opposite each presumed active zone is a postjunctional fold (Peper, Dreyer, Sandri, Akert & Moor, 1974) which can be made visible in the light microscope by cholinesterase staining (Pl. 1*A*). Visibility of the junctional folds was improved by denervating the muscles 15 days earlier. The spacing between adjacent folds (presumably reflecting release sites) was slightly less in the sartorius ($0{\cdot}74 \pm 0{\cdot}01$ μm, $n = 52$) than in the c.p. ($0{\cdot}77 \pm 0{\cdot}01$ μm, $n = 54$). While this difference was marginally significant ($P < 0{\cdot}05$), its direction is opposite that which would explain the observations. When normalized for mean sarcomere spacing in the particular fibres measured, these correspond to values of 3·3 folds/sarcomere in the sartorius, and 3·5 folds/sarcomere for the c.p. which, given the variability in measured values, we do not consider a statistically significant difference. We conclude that there is no apparent difference between the two muscles in numbers of presumed active zones per unit length of terminal. To determine whether the size of the active zone might be larger in the c.p. junctions, we compared the width of synaptic contact seen in randomly selected transverse sections through nerve terminals in the two muscles (Pl. 1*B*). There was no significant difference in apposition width between the sartorius ($1{\cdot}66 \pm 0{\cdot}07$ μm, $n = 97$) and the c.p. ($1{\cdot}52 \pm 0{\cdot}04$ μm, $n = 135$). Measurement of cross-sectional areas of the same terminals shows that the sartorius terminals were slightly larger ($1{\cdot}28 \pm 0{\cdot}10$ μm^2, $n = 97$), on the average, than c.p. terminals ($1{\cdot}01 \pm 0{\cdot}05$ μm^2, $n = 132$). If this difference in cross-sectional area were significant, the concomitant lower surface to volume ratio might result in a smaller increase in Ca^{2+} concentration within sartorius terminals for the same Ca^{2+} influx during the presynaptic action potential. However, the difference in cross-sectional area was only marginally significant ($0{\cdot}05 > P > 0{\cdot}025$) and thus clearly does not explain the difference in release observed.

Finally, counts of presynaptic vesicles and mitochondria were made in random e.m. cross-sections of terminals in both muscles (Pl. 1*B*). There was no significant difference in the number of vesicles in a standard $0{\cdot}33$ μm^2 area just above the presynaptic membrane (sart. $38 \pm 1{\cdot}4$, $n = 50$; c.p. $35{\cdot}5 \pm 1{\cdot}4$, $n = 82$). Although this may not be representative of the immediately releasable pool of transmitter, there was nevertheless no significant difference in vesicle density as measured in this way. Similarly, there was no apparent difference in the number of mitochondria per section through the terminal (sart. $4{\cdot}9 \pm 0{\cdot}3$, $n = 82$; c.p. $5{\cdot}0 \pm 0{\cdot}3$, $n = 98$) Thus, insofar as we measured, there was no significant difference in presynaptic morphology that would explain greater release from c.p. terminals. It seems probable that the difference is due to 'physiological' regulation.

Action potential invasion of the nerve terminal

One possible 'physiological' difference could be in the degree of invasion of the presynaptic action potential into the nerve terminal. If incomplete invasion of sartorius terminals were frequent, the amount of transmitter released might be much less than would be predicted from the morphology. Katz & Miledi (1965) showed, with extracellular recording, that the action potential actively propagates throughout the terminal in the frog sartorius. However, Wernig & Carmody (1978) suggested that invasion failure may occur in nerve terminals of the cutaneous pectoris. Because we felt that it was important to positively rule out this possibility, we checked indirectly for different degrees of terminal invasion in three ways. The first and most critical test was based on Poisson's theorem, which allows one to calculate the expected number of release failures when the probability of release is low (del Castillo & Katz, 1954). We looked for evidence of invasion failures in the most extreme cases, i.e. in seven sartorius end-plates with very low release per unit length ($\bar{x}$ = 0·17 qnanta/100 μm). If intermittent conduction failure occurred at these low quantal content junctions, then the observed number of e.p.p.s composed of one or two quanta would be fewer than would be predicted from the observed number of failures. For all the junctions studied, however, the number of single, double, and higher multiple quantal content e.p.p.s was as predicted from the number of failures, implying that these were actual Poisson release failures and not invasion failures. Furthermore, for each junction there was good agreement between quantal content calculated three different ways (Martin, 1966).

A second indirect method of testing for differences in terminal invasion took advantage of the observation in reinnervated end-plates that when one action potential fails to invade a terminal, a second, arriving shortly after, can lead to full invasion (Dennis & Miledi, 1974). Hence we looked for evidence of enhanced amplitude of the second of two e.p.p.s evoked 10 msec apart. If the second e.p.p. was clearly larger than could be accounted for by facilitation alone, as judged by comparison with similar measurements on c.p. junctions, then it might be suspected that a difference in degree of invasion might be responsible. Since facilitation varies inversely with quantal content (Hutter & Trautwein, 1956; Rahamimoff, 1968), we adjusted Ca^{2+} concentration between 0·25 and 0·3 mM such that quantal contents in junctions from the two muscles were approximately equal. Under these conditions there was no difference in apparent facilitation. The mean ratio of 2nd e.p.p./1st e.p.p. was 1·9, n = 11 (c.p.) and 2·0, n = 23 (sart.)

The third method of testing for differences in terminal invasion was based on a comparison of miniature end-plate potential frequency with quantal content and with terminal length at identified junctions (Fig. 4). If it is assumed that m.e.p.p.s are released with equal probability from all parts of the terminal, then m.e.p.p. frequency should provide a relative measure of total terminal length. If the action potentials characteristically invaded c.p. terminals more fully than sartorius terminals, then the relationship between quantal content and m.e.p.p. frequency should be different in the two muscles. As Fig. 4*A* shows, this was not the case. The same correlation between m.e.p.p. frequency and quantal content was seen for both muscles. Moreover, although the correlation between m.e.p.p. frequency and terminal length

is poor, the frequency per unit length was higher in the c.p. than in the sartorius (Fig. 4*B*), as expected from the difference in evoked release per unit length.

These arguments render unlikely the possibility that the difference in transmitter release is due to differences in terminal invasion by the action potential. We are left

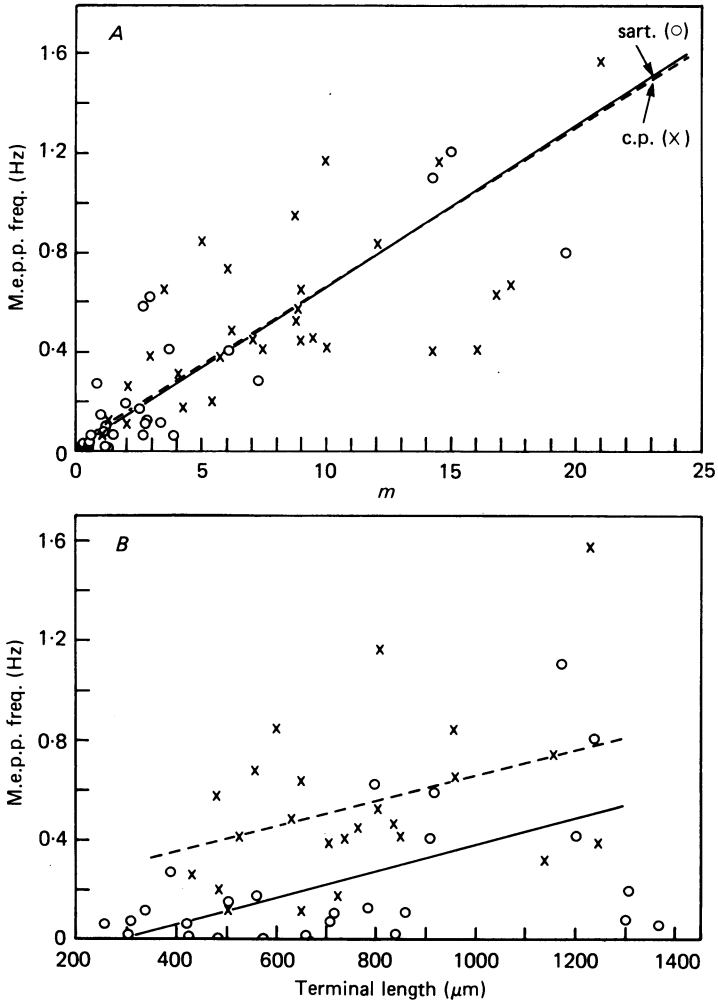

Fig. 4. Relationship between (*A*) m.e.p.p. frequency and quantal content, and (*B*) m.e.p.p. frequency and terminal length. Linear correlation coefficients for c.p. and sartorius respectively are: 0·71 and 0·98 in *A*, 0·38 and 0·60 in *B*.

with the conclusion that the probability of transmitter release is different in nerve terminals which, with present techniques, otherwise appear similar.

Comparison of the dependence of release on extracellular Ca^{2+}. We tested the possibility of one such physiological difference by examining the dependence of quantal content on external Ca^{2+} concentration for the two muscles. If the relationship between quantal content and external Ca^{2+} differed in slope, this might suggest a difference in the Ca^{2+} conductance of channels in the two types of terminal mem-

branes or that transmitter release was more sensitively coupled to internal Ca^{2+} in one case than the other. For each of several identified junctions, we measured quantal content at three different Ca^{2+} concentrations in the range 0·2–0·35 mM (Fig. 5). As expected from previous data, the curve for the c.p. is shifted to the left relative to that of the sartorius, but the slopes of the relationships are similar. This

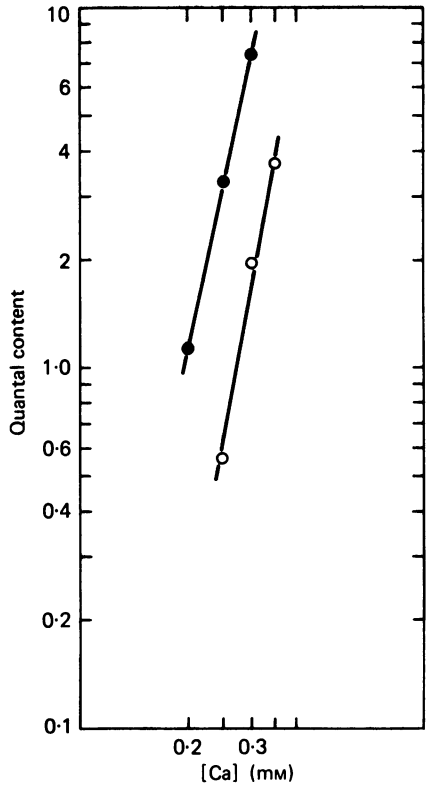

Fig. 5. Log-log plot of the effect of changing Ca^{2+} concentration on quantal content. Filled symbols, average values for a random sample of 18 c.p. terminals (linear correlation coefficient, $r = 0·99$; slope = 4·6). Open symbols, random sample of 8 sartorius terminals ($r = 0·99$; slope = 5·7).

implies that there is no significant difference in the kinetics of voltage-sensitive Ca^{2+} permeation or in the role of intracellular Ca^{2+} in triggering transmitter release.

Differences in physiological release mechanisms were further investigated by comparing the effect of depolarization on m.e.p.p. frequency at different external Ca^{2+} concentrations. From each of a series of identified junctions we recorded resting m.e.p.p. frequency in normal Ringer containing 2 mM-K^+ and elevated m.e.p.p. frequency in Ringer containing 10 mM-K^+. This was done in 0·3, 1·0, and 1·8 mM-Ca^{2+} for each junction. Tetrodotoxin (10^{-7} M) was present throughout to eliminate regenerative Na currents and prostigmine (10^{-6} g/ml.) added to make m.e.p.p.s more prominent. Thus the experiment was designed to test for differences in the role of voltage sensitive Ca^{2+} permeation in transmitter release. The results

are presented in Fig. 6. Consistent with the previously demonstrated difference in evoked release, at either K^+ concentration spontaneous release in c.p. terminals is greater than in sartorius terminals. In confirmation of the results of Mambrini & Benoit (1964) and Matthews & Wickelgren (1977), the lower two curves in Fig. 6*A*

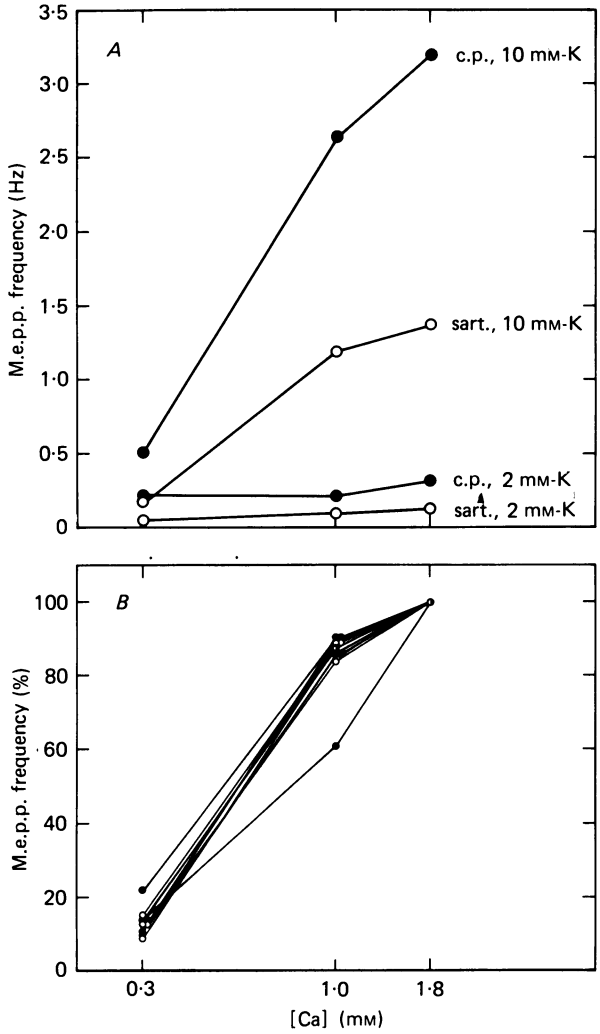

Fig. 6. *A*, the effect of changing $[Ca^{2+}]$ on m.e.p.p. frequency at c.p. (filled symbols) and sartorius (open symbols) end-plates in 10 mM-K (upper two curves) and 2 mM-K (lower two curves). Each c.p. point is an average of data from seven end-plates, while each sartorius point is an average of data from four end-plates. *B*, the effect of changing $[Ca^{2+}]$ on m.e.p.p. frequency at c.p. and sartorius end-plates. Symbols as in *A*. M.e.p.p. frequency is expressed as a percentage of the frequency in 1·8 mM-Ca^{2+}.

show that at normal K^+ concentration, m.e.p.p. frequency is only slightly dependent on Ca^{2+} concentration. When the terminal membrane is depolarized by high K^+, as in the case of the upper two curves in Fig. 6*A*, the dependence of m.e.p.p. frequency on Ca^{2+} concentration becomes more pronounced. Note that at any one Ca^{2+} con-

centration, the extent to which m.e.p.p. frequency in 10 mM-K^+ is elevated above that in 2 mM-K^+ is approximately the same for the two muscles. Thus what is presumed to be an equivalent depolarization leads to increases in release which are proportionately similar. Although in 10 mM-K^+ the absolute values of m.e.p.p. frequency are different in the two muscles, a normalized plot of m.e.p.p. frequency (Fig. 6*B*) shows complete overlap in nearly all cases.

DISCUSSION

The difference in safety factor observed for the sartorius and c.p. muscles can be seen to be explainable in part by differences in muscle fibre diameter and the resultant low R_{1n} for the larger sartorius fibres. However, an even more important difference is in the quantal content of junctions in each muscle, with much greater release at the c.p. terminals. For equivalent stretch of the muscle (1·1 times rest length) in Ringer containing 1 mM-Mg^{2+} and only 0·3 mM-Ca^{2+}, the mean quantal content at c.p. junctions was more than three times greater than at sartorius junctions. The difference in synaptic strength is most clearly demonstrated by comparing quantal release per unit length at identified terminals in the two muscles, where a 2·4-fold difference was observed when whole muscles were stretched to the same relative extent, and a 3·2-fold difference observed, in a smaller sample, when muscles were stretched to a constant sarcomere spacing of 2·4 μm. Presumably the difference between the 2·4-fold and the 3·2-fold values is explainable by the relatively limited sample size in both series of measurements. No obvious morphological correlates for the difference in release between the two muscles could be found, nor did a difference in degree of terminal invasion appear a likely candidate. It must be acknowledged, however, that our morphological analysis was by a random section through terminal regions, not by serial section and reconstruction of whole terminals.

The lack of a morphological correlate leaves us with the conclusion that some physiological difference must be responsible. There are many processes that could be implicated, e.g. the duration and amplitude of the presynaptic action potential, differences in the number or properties of voltage-dependent Ca^{2+} channels in the terminal membrane, the number of packets available for release, or some other feature of the release mechanism. Our experiments permit us to conclude that, with regard to both depolarization dependence and Ca^{2+} dependence, the properties of Ca^{2+} channels in terminals of both muscles are similar. The preparations behave as if the physiological difference between the two types of terminals were in the amount of available Ca^{2+} inside the terminal. This may be due to a difference in surface charge affecting access of Ca^{2+} to its channels in the terminal membrane, the density of Ca^{2+} channels in the membrane, the number of internal Ca^{2+} binding sites, or the level of Ca^{2+} sequestering activity.

Kuno *et al.* (1971) reported a positive but relatively poor correlation between terminal area, m.e.p.p. frequency, and quantal content. Similar results were found in the present study (Fig. 4*B*), suggesting that m.e.p.p. frequency and quantal content may also depend on factors other than terminal length, such as motor unit size or competitive interaction (see below).

We have expressed the difference in release properties of terminals in the two

muscles in terms of release per unit length. It should be noted, however, that release may not be uniform along the length of a terminal. Bennett & Lavidis (1979) have recently reported that, at low external Ca^{2+} concentrations, a large fraction of the transmitter released from motor nerve terminals in *Bufo marinus* may occur in a very restricted part of the terminal. If it is non-uniform, however, then our data suggest that c.p. terminals have more active release areas per unit length than do sart. terminals. Also, the good correspondence between m.e.p.p. frequency and quantal content in both muscles would indicate that spontaneous release of packets of transmitter occurs at the same sites as evoked release, in approximately the same proportion.

The present work confirms the sensitive dependence of transmitter release on the degree of stretch of the muscle. The dependence on stretch was found to be the same for both muscles, although the safety factors at c.p. junctions were so large in normal Ca^{2+} concentration that all the fibres contract even when the muscle is at rest length.

One can reasonably ask why the sartorius should normally have a population of muscle fibres that do not contract to a single stimulus. This is not the case in the c.p., and appears not to be the case in other vertebrate fast skeletal muscles studied. It may actually be an advantage to the animal, since it would allow change in the effective size of motor units as a function of the frequency of α-motoneurone discharge. At very low levels of nerve firing, a given motor unit would only be partially activated, and any contraction would be relatively weak. With increasing nerve activity, not only are additional motor units recruited, but individual motor units become more fully activated. Perhaps this is a mechanism for smoothing the control of contraction in large muscles. It would be of interest to determine whether this is the case for other large muscles in the frog. Perhaps the more valid question is why all-or-none contraction of motor units is such a common phenomenon in vertebrate skeletal muscle?

Finally, although we do not have an explanation for the difference in transmitter release per unit terminal length in the two muscles, it is of interest to formulate two alternative testable hypotheses for what might cause this difference. One hypothesis is based on the difference in motor unit size. Although both sartorius and c.p. nerves have similar numbers of motor axons, approximately ten to fifteen as judged by distinct increments in twitch tension with changes in stimulus intensity, the sartorius muscle is composed of slightly more fibres than is the c.p. (sartorius 911 fibres, c.p. 793 fibres, in one case). In addition, each sartorius fibre is innervated at two to five end-plates. Thus the number of end-plates per motor axon is at least 3 times higher in the sartorius. It can be postulated that for some reason it is not possible for a given frog motoneurone to maintain so many synapses with high levels of output. It might be that synapses of small motor units, independent of which muscle they are in, will prove to be stronger than synapses in large motor units. Preliminary data do indeed suggest that terminals in motor units experimentally reduced in size tend to release more transmitter per unit length than terminals in normal sized motor units in the sartorius (Herrera & Grinnell, 1979). A second hypothesis is that synapses on sartorius fibres are reduced in effectiveness due to some form of competitive interaction with other terminals in the same fibres. In this

sense the interaction might be equivalent to a mild form of the process of synapse elimination during development (Redfern, 1970) or to the phenomenon of synaptic competition observed between the axons of two foreign nerves implanted into frog muscle (Grinnell *et al.* 1977, 1979).

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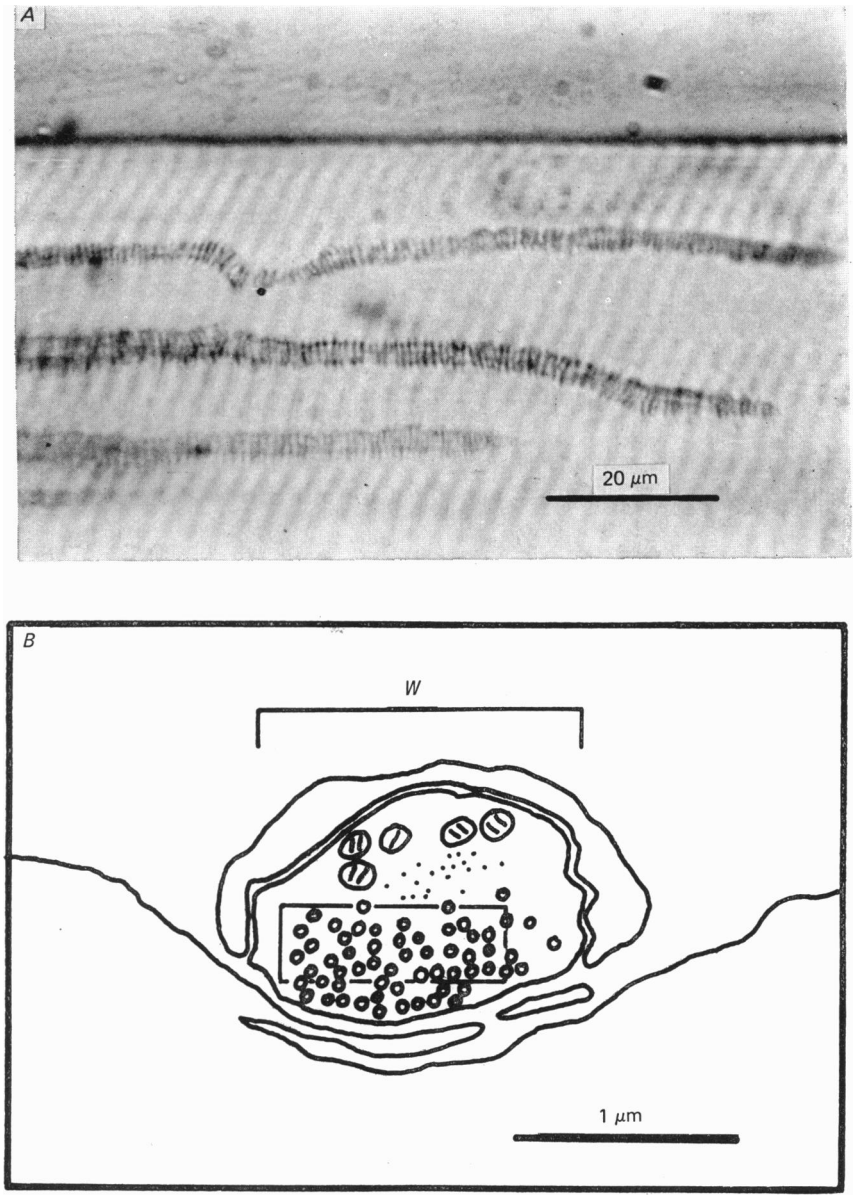

A. D. GRINNELL AND A. A. HERRERA

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EXPLANATION OF PLATE

A, light micrograph showing exposed junctional folds in a cholinesterase stained c.p. muscle. Muscle was denervated 15 days earlier. *B*, tracing from an electron micrograph showing how ultrastructural measurements were made. Rectangle shows the standard area within which vesicles were counted. Width of synaptic contact indicated by *W*.