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DUAL INNERVATION OF END-PLATE SITES AND ITS CONSEQUENCES FOR NEUROMUSCULAR TRANSMISSION IN MUSCLES OF ADULT *XENOPUS LAEVIS*

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SUMMARY

1. Electrophysiological study of dually innervated end-plate sites was carried out in *Xenopus laevis* pectoral muscle fibres. End-plate potentials (e.p.p.s) and miniature end-plate potentials (m.e.p.p.s) have been recorded in Mg-blocked preparations.

2. The mean quantal content (m) of each e.p.p. at dually innervated end-plates was significantly smaller than the corresponding values obtained at singly innervated ones. At a given doubly innervated end-plate site the values of m tended to be inversely related, so that the compound value of m (obtained by adding them) was in the same range as that found in singly innervated junctions. These findings were taken to suggest the existence of an upper limit in the average amount of transmitter released at a synaptic site.

3. It was found that neither intermittent presynaptic conduction block, nor particular muscle fibre properties could account for the low values of m in dual end plates. The small size of the nerve terminals appears to be the most likely explanation.

4. The sensitivity to acetylcholine and muscle fibre electrical properties were investigated; no differences were found between fibres with sub- or suprathreshold e.p.p.s.

5. The nature of the factors responsible for this presumed small size of the nerve endings (competition between nerve endings for a limited synaptic space on the muscle membrane or reciprocal interaction between closely located terminals) as well as the possible origins of polyinnervation are discussed.

INTRODUCTION

It is now well documented that developing vertebrate skeletal muscle possess polyneuronal innervation at single end-plate sites. By the end of the second week after birth in the rat (Redfern, 1970; Brown, Jansen & Van Essen, 1976) or on the completion of the metamorphosis in the frog (Letinsky, 1974; Bennett & Pettigrew, 1975) all but one of the nerve endings are eliminated and the end-plate sites become singly innervated. While in mammalian adult muscle single innervation of motor end-plates seems to be the rule (Brown & Matthews, 1960; Van Harreveld & Tachibana, 1961), recent reports have shown that polyinnervated end-plate areas are

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not uncommon in adult frog muscle (Dennis & Miledi, 1974; Rotshenker & McMahan, 1976; Vyskočil & Magazanik, 1977). Their incidence is around 25% of the total number of end-plates. In previous reports from this laboratory, polyneuronal innervation has been shown to exist in single end-plate sites of the pectoral muscle of *Xenopus laevis* (Daudin) although its incidence is much lower (Haimann, Mallart & Zilber-Gachelin, 1976; Mallart, Zilber-Gachelin, Haimann & Tomás Ferré, 1976). A quantitative analysis of transmitter release at doubly innervated synaptic sites has been performed and a comparison made with the release function at singly innervated sites. These data show in agreement with unpublished preliminary findings of Zilber-Gachelin, that neuromuscular transmission is less efficient at polyinnervated junctions. Possible explanations of the nature of synaptic depression and of the origin of polyinnervation in adult frog muscle will be discussed.

METHODS

Experiments were performed on the pectoral muscle of *Xenopus laevis*. For a detailed description of this preparation the reader should refer to Haimann *et al.* (1976) and Mallart *et al.* (1976). It will suffice to say here that the main particularity of this muscle is its dual innervation from two nerves (anterior and posterior nerves) so that about 20% of the muscle fibres are innervated by two axons from different nerves; in 25% of the dually innervated fibres both endings occur at the same synaptic site.

The muscle was bathed in a standard solution of the following composition (mM): Na, 118·0; K, 2·5; Ca, 1·6; H_2PO_4, 0·75; HPO_4, 2·25; Cl, 119·1; in which some of the Na was isosmotically replaced by 12 mM-Mg. The irreversible anticholinesterase *O*-ethyl *S*-2 di-isopropilaminoethyl methyl-phosphonothionate (Vigny, Bon, Massoulié & Leterrier, 1978) was applied once for 15 min at a concentration of 3×10^{-7} M before starting the experiment; the cholinesterase block was constant for at least 10 h. Collagenase (Sigma type III) at a concentration of 0·50 mg/ml. was applied for one hour to soften the conjunctive tissue; the risk of damage by mechanical dissection was thus avoided.

The nerves were stimulated using suction electrodes. 3 M-KCl filled intracellular electrodes with resistances of 5–8 MΩ were used for passing current or recording. Micropipettes filled with 2 M-acetylcholine of approximately 200 MΩ resistance were used for ionophoresis; current pulses (intracellular or ionophoretic) were delivered through a constant current source (Dreyer & Peper, 1974).

In an attempt to standardize the degree of neuromuscular block the same amount of Ca^{2+} and Mg^{2+} was used throughout the whole experimental series and the degree of stretch was adjusted so that the muscle did not twitch for a single stimulus but it did for a pair of stimuli delivered at 9 msec interval. In an attempt to get an unbiased sample of fibres with singly innervated end-plate sites they were always taken from the next one or two on either side of the fibre where a doubly innervated end-plate site was found.

The quantal content of the e.p.p. was estimated by dividing the mean e.p.p. amplitude by the mean m.e.p.p. amplitude and, in some cases, by the method of failures (del Castillo & Katz, 1954). Martin's equation was used to correct for non-linear summation of the e.p.p.s. since for slowly changing potentials (e.g. when using an anticholinesterase) membrane capacitance may be ignored (Adams, 1976).

Square-pulse analysis was performed by using 20 nA hyperpolarizing current pulses which gave (on the average) a 3–6 mV potential change. A drop in membrane potential was usually observed on insertion of the second electrode; to account for the shunt conductance when estimating input impedance, it was considered that the drop in membrane potential was proportional to the shunt of the membrane resistance, thus a proportionality factor was obtained which served to correct the measured input resistance. This analysis was performed on fibres of 15 mm long and the measured length constant was approximately 1·5 mm, infinite cable equations were therefore used (see Stefani & Steinbach, 1969).

RESULTS

Existence of doubly innervated end plate sites

Electrophysiological evidence of doubly innervated end-plate sites was obtained when two e.p.p.s with the minimal rise time could be recorded from the same electrode position on stimulation of both nerves (Fig. 1, inset). A more precise way to show the convergence of two endings on a single end-plate site is to displace the

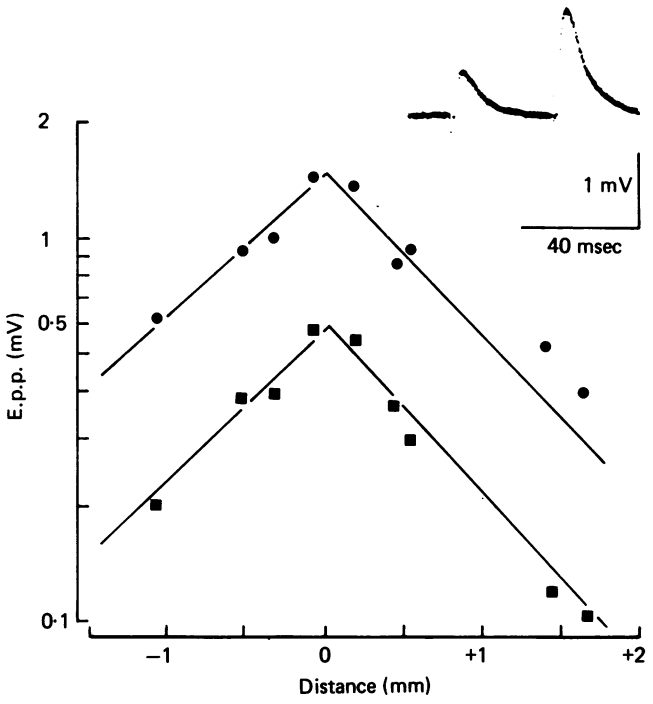

Fig. 1. Relation between the amplitude of e.p.p.s evoked by anterior (squares) and posterior (filled circles) nerve stimulation and the distance of the recording electrode from the focus (indicated here as O) in semilogarithmic coordinates. λ estimated from e.p.p. amplitude was 1·0 mm. Inset: example of e.p.p.s recorded near the focus on stimulation of anterior and posterior nerves (rise time = 2 msec for both responses). This trace is the average of 25 sweeps.

recording electrode in both directions along the fibre to get a measure of the electronic decay of e.p.p. amplitude with distance from the synaptic focus (Gage & McBurney, 1973) A precise location of the focus of the e.p.p.s elicited by stimulation of each nerve can thus be obtained. In the example given in Fig. 1, it can be seen that both foci are located exactly at the same point on the muscle fibre. No end-plate sites innervated by more than two axons have been found. Their existence cannot be excluded, however, since changes in stimulus intensity or duration, required to reveal the presence of axons with different threshold, has not been performed in all the cases. For the same reason, the number of doubly innervated end-plate sites from the same nerve found in this study (see Fig. 4, inset) may represent an underestimation of their true incidence. In eight fibres out of thirty-

six a singly innervated end-plate site was found to exist at a distance of approximately 2 mm from the doubly innervated one. Although a careful analysis of this type of innervation could not be performed due to the small size of the sample, it appears, however, that no interaction seems to exist between distant synapses on the same muscle fibre.

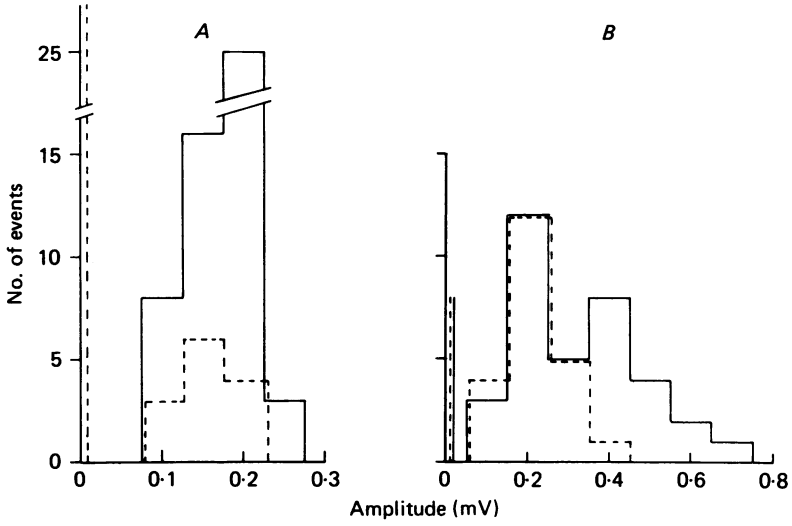

Fig. 2. Amplitude histograms of e.p.p.s and m.e.p.p.s recorded at two dually innervated end-plate sites. *A*, amplitude histogram of e.p.p.s evoked by anterior nerve stimulation (dashed line) compared to amplitude histogram of miniature potentials (continuous line). *m* of anterior nerve e.p.p. calculated from the method of failures was 0·35 and that of posterior nerve e.p.p. calculated by the direct method was 32·6. B, amplitude histograms of e.p.p.s evoked by anterior (continuous line) and posterior (dashed line) nerve stimulation. *m* of anterior and posterior nerve e.p.p.s calculated from the method of failures were respectively 1·70 and 1·32. The vertical lines near the abscissae origin represent the number of failures.

Quantal content of the e.p.p.s elicited at dually innervated end-plate sites

The identity of the sizes of the quanta released by both endings had to be established before performing quantal analysis of the e.p.p.s evoked by stimulation of either nerve. Two procedures were used for this purpose depending on the amplitude of the e.p.p.s recorded at a given end-plate site. (1) When the stimulation of one nerve evoked a large amplitude e.p.p. and the other a very small one, it was assumed that the great majority of m.e.p.p.s corresponded to quanta released by endings of the nerve that gives the larger e.p.p. (see Fig. 5) and the small amplitude e.p.p.s were compared to m.e.p.p.s. amplitude (Fig. 2*A*). (2) When the stimulation of both nerves elicited small e.p.p.s. of unit size, their amplitudes were compared (Fig. 2*B*). One can thus conclude that each quantum of acetylcholine released by either nerve at doubly innervated end-plate sites evokes the same amount of depolarization at the post-synaptic membrane.

The quantal content of the e.p.p.s in a sample of thirty-six doubly innervated end-plates from twenty-six muscles was compared with that of 53 singly innervated

end-plates from the same muscles. Since two e.p.p.s could be recorded from the doubly innervated sites two values of m corresponded to each of these sites. The frequency distribution of the values of m in both samples is represented in Fig. 3. It can be seen that although m was in the same range in both populations, the cases in which m was less than 5 predominate overwhelmingly in doubly innervated end-plate sites. To carry out a more refined analysis of the distribution of m, doubly innervated end-plate sites have been regarded not only as a synaptic area with two inputs where two independent values of m can be obtained, but also as a single synapse for which a compound value of m can be obtained by adding both values of m.

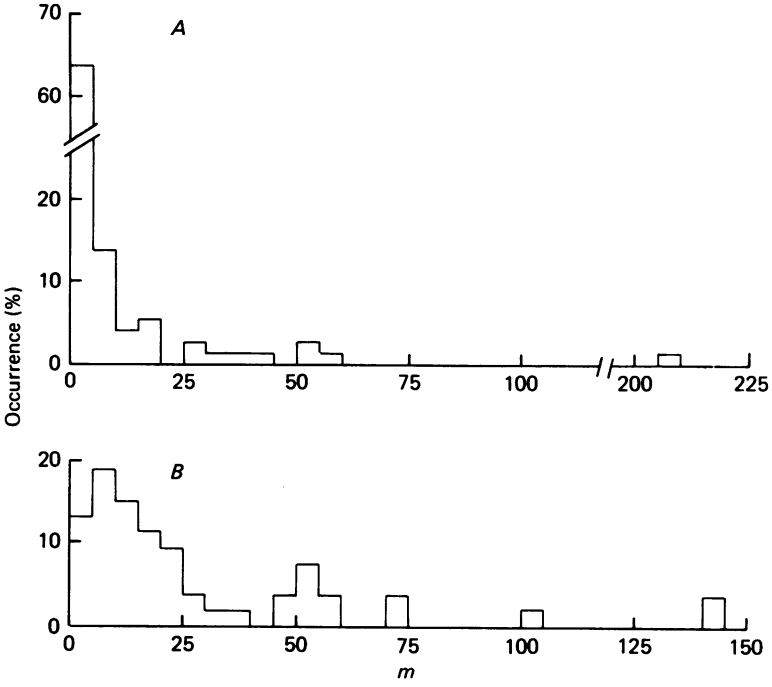

Fig. 3. Distribution of the value of m for e.p.p.s recorded at dually (A) and singly (B) innervated end-plate sites. In A the sample was seventy-two values of m obtained from thirty-six fibres and in B fifty-three values of m obtained from an equal number of fibres.

Since, as can be appreciated from Fig. 3, the values of m showed a skew frequency distribution, the median rather than the arithmetical mean, and the interquartile range, rather than the standard deviation were chosen to represent the average and the scatter of the values of m. The smaller and the larger values of m, as well as the compound value in each doubly innervated end-plate site were treated separately and their median and interquartile range calculated. These data are shown in Table 1 together with those obtained from singly innervated end-plate sites. These figures reveal that significant differences in m may exist between singly and doubly innervated end-plates even when m of the former is compared to compound m of the latter. The Mann-Whitney U test (Siegel, 1956) was therefore used

TABLE 1. Median and interquartile range of the values of m in doubly and singly innervated end-plate sites

Doubly innervated end-plate sites (n = 36)			Singly innervated end-plate sites (n = 53)
Larger e.p.p. in a fibre	Smaller e.p.p. in a fibre	Large + small e.p.p.s in a fibre	
9·4 (5·0–26·5)	1·0 (0·5–1·4)	9·6 (5·4–19·8)	16·4 (7·2–40·5)

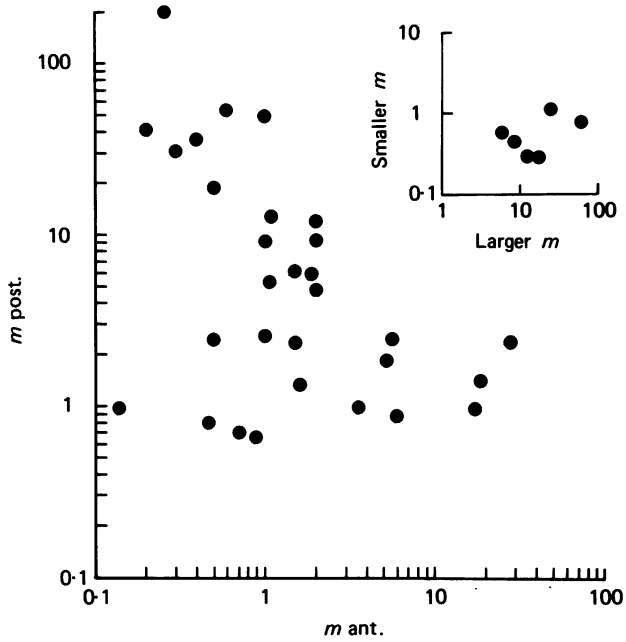

Fig. 4. Relation (in double logarithmic co-ordinates) between the quantal content of the two e.p.p.s recorded at dually innervated end-plate sites on stimulation of either nerve. Inset represents the relation between the quantal contents of the two e.p.p.s when the two axons innervating the end-plate are supplied by the same nerve.

to test whether the apparent differences in m were statistically significant. It was found that the compound value of m from doubly innervated end-plate sites is significantly smaller than the m found at singly innervated junctions (P = 0·06); the larger m from dual junctions was significantly smaller than the m in single ones (P < 0·05), and, as expected, the difference between the latter and the small m of dual junctions was highly significant (better than P < 0·0001).

At a given dual end-plate site, the e.p.p.s elicited from different axons were not, as a rule, of similar quantal content, particularly when the value of m from one of the nerves was larger than about 5, in which case the values of m tend to be inversely related. This is shown in Fig. 4 from which it appears that a large response from one nerve was frequently accompanied by a small response from the other. When both endings gave rise to responses of similar m, these are of medium or small

amplitude. Cases in which both responses were of large amplitude have never been observed. These observations suggest that, for a given muscle, there is an upper limit in the average amount of transmitter that can be released at each synaptic site and that this limit can be reached either by one or by more axons.

The question now arises of what are the factors responsible for this limitation in transmitter output in doubly-innervated end-plate sites. Among many possible causes we shall consider first an eventual intermittent failure of presynaptic action potentials to invade some terminal branches and, second, a possible small size of the muscle fibre which in turn would lead to small end-plates and to e.p.p.s with low *m* (Kuno, Turkanis & Weakly, 1971; Bennett & Raftos, 1977).

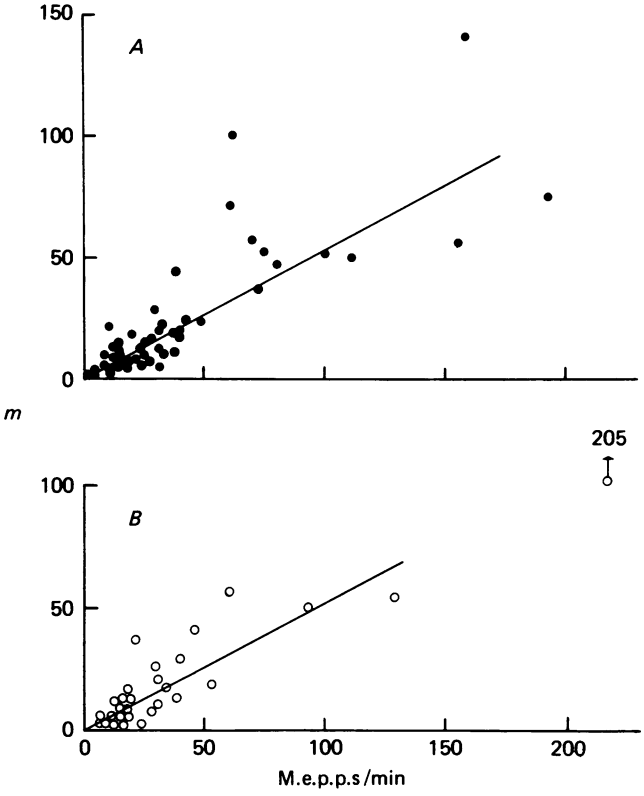

Fig. 5. Relation between the frequency of m.e.p.p.s and *m* in singly (*A*) and doubly (*B*) innervated end-plate sites. In *B*, the ordinate indicates the compound value of *m*. The Spearman rank correlation coefficient was 0·85 in *A*, 0·90 in *B*.

Intermittent presynaptic conduction failure is not the cause of the low values of m

Reduced transmitter output at dually innervated sites might result from intermittent conduction block at the branching points of the terminal arborizations (Bittner & Harrison, 1970; Hatt & Smith, 1976). Unpublished results from this laboratory showed that this phenomenon is a common feature of the regenerating neuromuscular junctions of *Xenopus* pectoral muscle: e.p.p.s had a polyphasic shape or showed multiquantal jumps in amplitude and latency from one stimulation

to another. These components could not be separated by changes in stimulus intensity.

In principle, we cannot exclude the existence of a presynaptic branch block in some of the normal adult neuromuscular junctions (Wernig & Carmody, 1977). An easy way to detect its presence is to compare the evoked with the spontaneous quantal release at a given junction. Since it has been shown by Kuno *et al.* (1971) that there is a good correlation between m.e.p.p. frequency and m, a reduction in the latter not paralleled by a change in the former would reveal a presynaptic conduction block in some of the branches. Miniature frequency and m were compared and the results are shown in Fig. 5, where m is plotted against miniature frequency in singly (A) and doubly (B) innervated end-plate sites. A good correlation was found to exist in both cases with a Spearman rank correlation coefficient of 0·85 and 0·90 respectively (Siegel, 1956). The slope of the least-squares regression line was 0·52 in singly and 0·51 in doubly innervated end-plate sites. If the incidence of the propagation failures were greater at doubly than at singly innervated sites, a comparatively smaller slope ought to be expected in the latter. Since this was not the case, one can conclude that, if propagation failures do occur, they are as frequent in one case as in the other.

A more precise way to detect the existence of a terminal branch block is to compare the observed and the expected number of e.p.p. failures; these ought to agree only if the failures were of release rather than of propagation. The expected number of failures was calculated from the Poisson law (del Castillo & Katz, 1954) in a low m sample from our population of doubly innervated end-plate sites, on the assumption that for values of $m \simeq 1$ the amplitude distribution of the e.p.p.s can be described by Poisson statistics (Wernig, 1975). The paired Student t test did not show any significant differences between the observed and the expected number of failures. These results favour the hypothesis of small size nerve terminals rather than intermittent presynaptic block as an explanation for the low values of m.

Differences in membrane physical constants cannot account for the low values of m in dual end-plates

Since end-plates of small size are more likely to be found in small diameter muscle fibres (Kuno *et al.* 1971) it seemed worth to investigate whether doubly innervated end-plate sites were formed preferentially on fibres in the lower diameter range.

Muscle fibre diameter and other membrane constants were calculated from data obtained by square pulse analysis (Fatt & Katz, 1951). Myoplasm resistivity (R_i) was taken as 170 Ωcm according to the estimation obtained in *Xenopus* muscle by Nakajima & Bastian (1974). To test the validity of this figure and of all the assumptions on which the present square-pulse analysis has been based (see Methods), a comparison was made of the calculated and the measured diameters. The latter were obtained from the superficial layer of fibres of a muscle frozen in liquid nitrogen, sectioned in a cryostat and stained for succinic acid dehydrogenase. The diameter of each muscle fibre was estimated from the mean of two measurements at right angles. Fig. 6 shows that the calculated values are in good agreement with those obtained by optical measurements providing thus a good support to our basic assumptions.

The relationship between m and muscle fibre diameter is illustrated in Fig. 7.

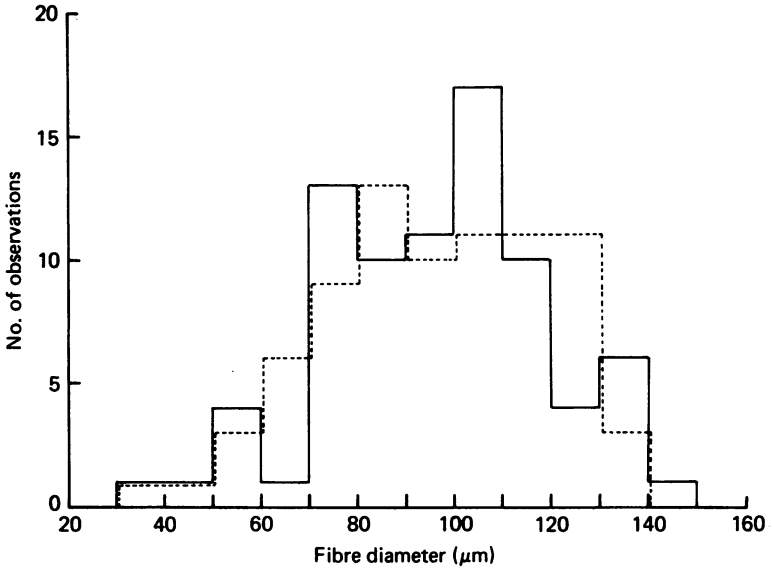

Fig. 6. Distribution of diameters of pectoral muscle fibres obtained either by the cable theory equation (continuous line) or by optic measurement (see text) (dashed lines). The cable equation was applied to seventy-nine fibres from twenty-six muscles. The optical measurements were performed on seventy-nine fibres from the superficial layer of a single muscle; this sample represents all the fibres in this layer except for a few at both edges of the muscle.

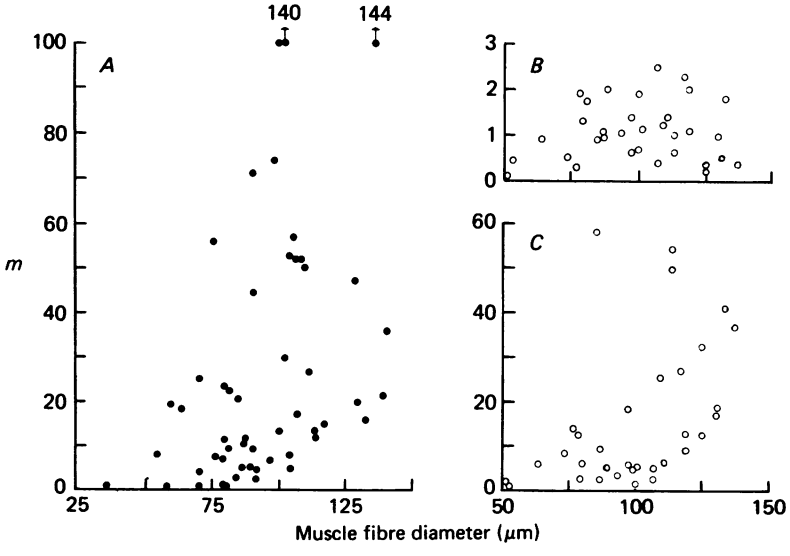

Fig. 7. Relation between m and muscle fibre diameter in singly (A) and dually innervated end-plate sites (B and C). B corresponds to the smallest and C to the largest values of m in each pair of values. The Spearman rank correlation coefficient was 0·48 ($P < 0·02$) in A and 0·52 ($P < 0·01$) in C, while it was 0·04 in B.

Although there is a tendency for large values of m from singly innervated end-plate sites to be found in fibres of large diameter (Fig. 7*A*), a Spearman rank correlation coefficient of 0·48 indicates that no good correlation exists between these two parameters. If we treat separately the larger and the smaller value of m in each doubly innervated end-plate site, we can see that the group of the large values of m resembles that of singly innervated junctions (the Spearman rank correlation was 0·52) (Fig. 7*C*). On the contrary, in the group of the small values of m absolutely no correlation exists between m and muscle fibre diameter (Fig. 7*B*). Since, as stated before, a doubly innervated end-plate area may also be regarded as a single synapse with a compound value of m, one might expect to find a better correlation between this value of m and fibre diameter. This was not the case and the obvious explanation was that the small e.p.p.s had very small values of m distributed over the whole range of diameters (Fig. 7*B*). These results enable one to rule out the hypothesis of a causal relationship between the small values of m in doubly innervated end-plate sites and muscle fibre diameter.

TABLE 2. Mean values and standard deviations of membrane constants of fibres with singly (*S*) and doubly (*D*) innervated end-plate sites

	Diameter (μm)	λ (mn)	R_m (Ω cm^2)	τ_m (msec)
S	93·8 ($\pm$ 22·6)	1·69 ($\pm$ 0·3)	2219 ($\pm$ 947)	19·4 ($\pm$ 9·5)
	n = 45	n = 45	n = 45	n = 17
D	99·0 ($\pm$ 22·5)	1·78 ($\pm$ 0·3)	2390 ($\pm$ 1265)	15·8 ($\pm$ 5·7)
	n = 34	n = 34	n = 34	n = 17

Kuno *et al.* (1971) have shown that a reasonably good correlation exists between end-plate area measured by the cholinesterase reaction and muscle fibre diameter. The present results indicate, however, that a poor correlation or no correlation at all exists between m and fibre diameter. The contradiction is only apparent if one considers that end-plate area, measured by the cholinesterase method, may not necessarily reflect the actual size of the nerve endings. If this is the case, m may depend closely on the size of the nerve endings (or, more precisely, on the number of release sites, Wernig, 1975) without being related to end-plate area or muscle fibre diameter. We shall discuss later the implications of this view.

Membrane electrical constants have been calculated in the two populations of muscle fibres and the data concerning space constant (λ), specific membrane resistance (R_m) and time constant (τ_m) are presented in Table 2. No significant differences between the two groups have been observed.

It must be noted that the values of λ obtained from the e.p.p. (Fig. 1) are substantially shorter than those obtained from square-pulse analysis because in the former case, the membrane has no time to reach its full charge (Katz, 1966; Gage & McBurney, 1973). Shorter values of λ (0·7–0·8 mm) have been observed in this muscle when curare instead of Mg^{2+} was used as a blocking agent (T. Gordon & A. Mallart, unpublished). The reason for this is the shortening of the end-plate current produced by curare (Mallart & Molgó, 1978).

Hyperinnervation of end-plate sites and muscle fibre activity

Since it has been shown that muscle activity plays a role in the elimination of redundant synapses in neonatal muscle (Benoit & Changeux, 1975), and that, on the contrary, the lack of this activity can create suitable conditions for the estabishment of supernumerary endings (Fex, Sonesson, Thesleff & Zelená, 1966; Jansen, Lømo, Nicolaysen & Westgaard, 1973; Lømo & Slater, 1978), one may hypothesize that doubly innervated junctions exist because of the lack of propagated muscle activity.

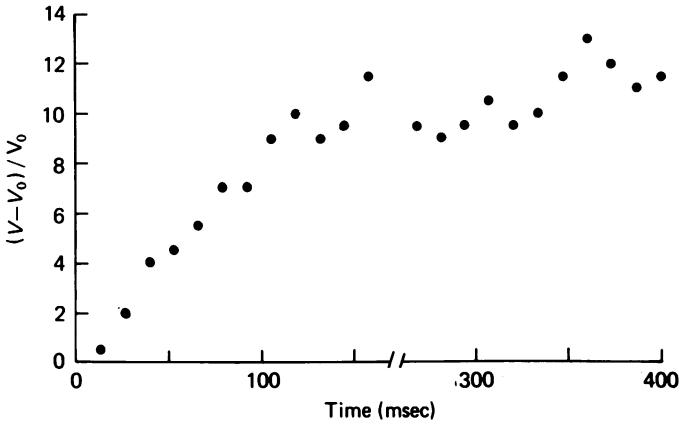

Fig. 8. Facilitation of e.p.p.s recorded at a dually innervated end-plate site during a 400 msec train of stimuli at 13 msec interval delivered to th anterior nerve. The bathing solution is normal Ringer to which 4×10^{-6} M tubocurarine has been added. m of anterior and posterior nerve e.p.p.s calculated from the method of failures were 0·92 and 0·67 respectively in a 12 mM-Mg Ringer solution. Abcissa indicates time after the first shock in the train. Ordinate indicates facilitation defined by $(V - V_0)\ V_0$, where V is the amplitude of a test response and V_0 is the amplitude of the response to the first shock. Because of movement artifact the top of seven e.p.p.s in the middle of the train fell off the oscilloscope screen.

From the fact that in more than half of the doubly and in a much smaller fraction of the singly innervated junctions values of $m < 10$ were found in a 12-mM-Mg bathing solution it seemed not too unrealistic to consider that a certain degree of inactivity could exist in their corresponding muscle fibres. For instance, by taking 0·2 mV as the average amplitude of the quantum of acetylcholine in this muscle, the depolarization produced by 10 quanta would be 2 mV which in normal saline would give an e.p.p. of about 20 mV (del Castillo & Engbaeck, 1954) which is unlikely to trigger a muscle action potential.

Muscle fibres which had junctions with $m < 5$ (to be on the safe side) were therefore investigated for inactivity-dependent changes. The exploration of the sensitivity to ionophoretic acetylcholine showed that at 1 mm on either side of the junction the sensitivity to acetylcholine fell by four orders of magnitude; a similar gradient was found in normal muscles with suprathreshold e.p.p.s. An increase in specific membrane resistance (R_m) has been shown to occur in denervated muscle (Hubbard, 1963) but, as shown by the data in Table 2, no difference exists in R_m between fibres with singly or doubly innervated end-plate sites.

The possibility exists that very small e.p.p.s may become suprathreshold in the intact animal when the motoneurones fire repetitively. In a series of experiments very small e.p.p.s were sought in 12 mM-Mg Ringer solution. Their amplitude was measured again in normal Ringer and then blocked by adding 4×10^{-6} M tubocurarine to the normal saline. The nerve was stimulated with a train of 200 msec at 75 Hz; this frequency is close to the maximal rate of firing of frog motoneurones (Magherini, Precht & Schwindt, 1976). In the experiment illustrated in Fig. 8 the mean amplitude of the e.p.p. was 0·2 mV in Mg-Ringer, 2·8 mV in normal Ringer solution and reached a 12 times larger amplitude upon facilitation by repetitive stimulation. This amplitude would have been large enough to trigger an action potential if no tubocurarine had been added to the bath. From the results obtained with this type of experiment it is possible to infer that propagated electrical activity can be generated, under certain circumstances, at doubly innervated end-plate sites. These data, together with the fact that some dual end-plates display large values of m (see Figs. 3 and 4), are taken to indicate that the presence of polyneuronal innervation in this muscle is unlikely to be related to the absence of propagated electrical activity.

DISCUSSION

The main finding of this research is that in cases where two different axons form synaptic contacts at the same end-plate site the amount of transmitter released by each ending is smaller than that released at singly innervated end-plate sites.

From the close relationship that exists between m and miniature frequency at doubly innervated end-plate sites, and from the agreement between the observed and the expected number of failures, it has been concluded that intermittent presynaptic failure of invasion of the terminals is not the cause of this reduced evoked transmitter release. Smaller values of m together with low miniature frequency would rather indicate small size terminals. This may in turn be due to small muscle fibre diameter (Kuno *et al.* 1971); but since no significant difference has been observed in diameter or in other physical constants between fibres with singly or doubly innervated end-plate sites one can rule out this possibility.

Where does this limitation in size of the terminals come from? In principle there is no obvious reason for nerve endings not to reach their full development as is usually found in singly innervated end-plate sites. Two mechanisms can be considered to explain it. One would be the existence of a competition between endings for a limited synaptic space on the muscle membrane, the other a reciprocal interaction between closely located terminals.

It is possible that doubly innervated end-plate sites represent a case of persistence of the polyneuronal innervation found in developing muscle (see Letinsky, 1974 and Bennett & Pettigrew, 1975 for a description in amphibia) where endings develop with spatial constraints in such a way that the total synaptic area formed either by one or more endings must lie (on the average) within fixed limits (see Mallart, 1977). This implies that, in a given muscle fibre type, all the synaptic areas formed would not be significantly different from each other whether being innervated by one or more axons. A more likely explanation would be, as suggested by Brown & Ironton (1978), that occasionally a single nerve terminal does not occupy all the available

synaptic space on the fibre membrane of adult muscle, thus allowing sprouts from a neighbouring axon to form synaptic contacts in close vicinity. This has been shown to occur in reinnervating muscle of amphibia (Rotshenker & McMahan, 1976; Haimann *et al.* 1976; Mallart *et al.* 1976; Grinnell, Rheuben & Letinsky, 1977), of mammals (Hoffmann, 1951; Brown & Ironton, 1978) and in an unoperated frog muscle after a contralateral denervation (Rotshenker & McMahan, 1976). Evidence has been obtained by Barker & Ip (1966) and Tuffery (1971) in the cat and by Pecot & Wernig (unpublished) in the frog that in normal adult muscle the motor endings are not static, but rather they are subjected to a continuous process of retraction and sprouting. The distribution of extrajunctional responses to ionophoretic acetylcholine in strips that seem to prolong the nerve terminals (Feltz & Mallart, 1971) probably correspond to former synaptic areas left vacant by the terminals. In this way, the portion of post-synaptic membrane made available by the retraction of one of more terminal branches can be occupied by terminals from the same or from a different nerve. Whatever the cause may be – persistence of a neonatal condition or turnover of endings – one can regard the smaller size of the terminals at doubly innervated end-plate sites as the result of a competition between nerve endings for a limited synaptic space. Cases may exist in which the space left vacant by the retraction of some of the terminal branches has not been completely occupied by the sprouts from another axon. In this case, the compound value of m of double end-plates would be, on the average, less than the value of m at singly innervated junctions. A tendency in this direction has been observed in our experiments; this is made apparent when comparing both median values (Table 1). However, both populations were statistically different only at $P = 0{\cdot}06$ according to the Mann-Whitney U test.

Another explanation for the small size of the terminals may be that each motor axon plays a role in the limitation of the sprouting of neighbouring axons. According to observations obtained in cutaneous receptive fields of salamanders (Aguilar, Bisby, Cooper & Diamond, 1973; Diamond, Cooper, Turner & Macintyre, 1976 and Cooper, Diamond & Turner, 1977) the target tissue promotes a sprouting of nerve endings that can be halted by inhibitory agents released by neighbouring terminals. Such an action can be exerted between axons when they form synapses in close vicinity. This situation exists in this muscle. According to silver impregnations performed by Tomás-Ferré (1977) in *Xenopus* muscles, the two axons that converge upon doubly innervated end-plate sites either form endings side by side few micrometres apart or give terminal branches that cross each other. This situation is different to what occurs in frog muscle where the two axon terminals run together in the same gutter (Rotshenker & McMahan, 1976). Preliminary observations from this laboratory show that the interaction between endings is not exerted at distances of about 2 mm or more. This is in agreement with recent data reported by Kuffler, Thompson & Jansen (1977), that the elimination of synapses depends on distance between end-plates.

The functional consequence of close range hyperinnervation is that often one or both endings release less transmitter than average giving only subthreshold e.p.p.s. In many cases, the muscle fibre discharges action potentials only when the e.p.p.s are facilitated by a train of stimuli. Since these fibres are likely to be less active one

would be tempted to conclude that this situation makes them more receptive to supernumerary innervation. In this respect, it is worth noting that M. Pecot & A. Wernig (unpublished) have observed a higher incidence of retraction and sprouting of nerve endings in chronically curarized frogs as compared to normals. However, until more evidence is made available on the relationship between activity and muscle membrane properties in the frog (see Letinsky, 1975) one cannot relate the cases of hyperinnervation observed here to the degree of muscle activity. The presence of doubly innervated end-plate sites must then be regarded as a phenomenon related to the normal process of retraction and sprouting of the terminals.

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