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BIMODAL MINIATURE AND EVOKED END-PLATE POTENTIALS IN ADULT MOUSE NEUROMUSCULAR JUNCTIONS

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SUMMARY

1. Intracellular recordings of spontaneous miniature end-plate potentials (m.e.p.p.s) in muscles from adult CBF-1 mice revealed a population of muscle fibres in which the amplitude distribution of m.e.p.p.s was bimodal. The large mode m.e.p.p.s were similar to those from fibres having unimodal amplitude distributions and the small mode m.e.p.p.s were about one-half to one-quarter the amplitude of the large mode.

2. In five diverse muscle groups (extensor digitorum communis, gluteus maximus, diaphragm, extensor digitorum longus, and soleus) from mice 10–12 or 31 months of age, bimodal m.e.p.p. amplitude distributions were present in about 20 % of fibres sampled.

3. In the common bimodal distribution (type 1), the rise times of small mode m.e.p.p.s were similar to those of large mode m.e.p.p.s. A rare class of small mode m.e.p.p.s (type 2) having long rise times was also observed.

4. Amplitudes and half-decay times of type 1 small mode m.e.p.p.s increased in the presence of an anticholinesterase (edrophonium). Increasing extracellular potassium concentration led to an increase in large mode m.e.p.p. frequency but had more variable effects on small mode frequency. In the few cases available for study, type 2 small mode m.e.p.p.s disappeared after addition of edrophonium or increased potassium.

5. When the extracellular calcium/magnesium ratio was reduced, large mode but not small mode m.e.p.p. frequency decreased.

6. In almost all muscle fibres in which end-plate potentials (e.p.p.s) were evoked by nerve stimulation at 20 Hz in low calcium/high magnesium solution, small mode e.p.p.s similar to small mode m.e.p.p.s appeared during 'failures' of large mode m.e.p.p.s. Also, in twelve out of fifteen fibres which had unimodal m.e.p.p. amplitude distributions, small mode e.p.p.s appeared which were similar in amplitude to small mode m.e.p.p.s in fibres with type 1 bimodal m.e.p.p.s.

7. Thus, if both spontaneous and evoked potentials are included, small mode m.e.p.p.s are present at most CBF-1 mouse adult neuromuscular junctions indepen-

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dent of muscle type or animal age. Small and large mode m.e.p.p.s differ in certain responses but both are evoked by nerve stimulation at physiological frequencies and therefore participate in normal neuromuscular synaptic activity. The possible origin of small mode m.e.p.p.s is discussed.

INTRODUCTION

Subminiature miniature end-plate potentials (m.e.p.p.s) recorded at vertebrate neuromuscular junctions form a distinct population of spontaneous potentials with amplitudes at least 2–3 times smaller than those of the more commonly described m.e.p.p. For the most part, subminiature m.e.p.p.s have been reported under conditions in which the motor nerve terminal is developing, relatively immature, or regenerating (e.g. Dennis & Miledi, 1974; Letinsky, 1974; Bennett & Pettigrew, 1975; Kriebel, Llados & Matteson, 1976) or after exposure to botulinum toxin (Harris & Miledi, 1971; Cull-Candy, Lundh & Thesleff, 1976; Kriebel *et al.* 1976). The available descriptions of subminiature m.e.p.p.s in *adult* muscle are derived from selected subpopulations of small fibres (Bevan, 1976) or from cases in which the nerve was subjected to exhaustive and non-physiological stimulation (Cooke & Quastel, 1973; Kriebel, 1978). Thus, it is unclear whether subminiature m.e.p.p.s are characteristic only of immature or pathologically altered nerve terminals, or whether they comprise a normal component of transmitter release. In addition, it is controversial (in the frog) whether subminiature end-plate potentials (e.p.p.s) can be evoked by nerve stimulation (compare Bevan, 1976 to Kriebel, 1978). Further, since there are structural differences in nerve terminals in different muscle types (e.g. slow *vs.* fast twitch muscle, Ellisman, Rash, Staehelin & Porter, 1976), there might be corresponding differences in the prevalence of subminiature m.e.p.p.s. Lastly, if subminiature m.e.p.p.s were mainly a phenomenon associated with development or nerve terminal plasticity (Bevan, 1976), a low incidence would be encountered in adult muscles and a still lower prevalence in muscles of old animals, in which it has been proposed that plasticity is reduced (Gutmann & Hanzlikova, 1972).

In the course of an extensive study of m.e.p.p.s in a variety of muscle types from young and old mice, we observed many muscle fibres with bimodal m.e.p.p. amplitude distributions, and thus were able to investigate the incidence and certain characteristics of these bimodal m.e.p.p.s. Surprisingly, the *majority* of fibres with unimodal spontaneous m.e.p.p. distribution yielded e.p.p. histograms with a small mode peak when the nerve was stimulated at physiological frequencies under the appropriate conditions. Other unusual responses of the small mode m.e.p.p.s further distinguished them from 'classical' large mode m.e.p.p.s.

METHODS

Male CBF-1 mice (Charles River Laboratory) were anaesthetized with methoxyflurane (Pitman-Moore). Muscles (usually soleus and diaphragm) were removed and pinned out on Sylgard (Dow Corning) in modified Krebs saline (pH 7·2) of the following composition (mM): NaCl, 135; KCl, 5; $NaHCO_3$, 15; Ca gluconate, 2·5; $MgSO_4$, 1; Na_2HPO_4, 1; glucose, 11. A gas mixture of 95% O_2 and 5% CO_2 was bubbled through the saline which was maintained at 30·0 ± 0·5 °C for all experiments. In order to record evoked e.p.p.s, a low calcium (0·4 mM)/high magnesium (2·75 mM) Krebs saline

was used and the nerve was stimulated supramaximally at a frequency of 20 Hz. In some experiments, 'cut fibre' preparations were prepared as described elsewhere (Banker, Kelly & Robbins, 1983).

Conventional glass capillary micro-electrode techniques (Banker *et al.* 1983) were used to make intracellular recordings of spontaneous m.e.p.p.s and e.p.p.s. Potentials were recorded on a Hewlett-Packard 3964A instrumentation recorder and later played back via an analog-to-digital converter into a PDP 11/23 minicomputer which was used to correct amplitudes to a standard resting membrane potential −80 mV (Kelly, 1978). Recording noise levels were between 0·1 and 0·15 mV peak-to-peak and hence the threshold for acceptance of m.e.p.p.s was usually between 0·12 and 0·18 mV depending on the signal-to-noise ratio. The computer analysis provided values for amplitude, frequency, rise time and half-decay time of m.e.p.p.s as well as amplitude of e.p.p.s. The Mann–Whitney non-parametric test was used to determine significance ($P < 0·05$) of differences between groups of data.

In some experiments, the electrode recording m.e.p.p.s was maintained inside the muscle fibre while the bathing solution was changed to one containing the cholinesterase inhibitor, edrophonium (Tensilon, Roche Laboratories). In experiments with high potassium solutions, the yield of successful recordings was increased by adding concentrated KCl to the bath to reach a final concentration of approximately 10–15 mM in 1–2 min without change of solution. In order to examine muscle fibres for the existence of dual end-plates, single dissociated fibres were stained for cholinesterase by methods detailed elsewhere (Robbins, Olek, Kelly, Takach & Christopher, 1980).

RESULTS

Occurrence and description. Bimodal m.e.p.p. amplitude distributions were observed with a frequency of about 20 % in all five muscle types from both young and old mice (Table 1). The amplitude of the smaller mode m.e.p.p. was usually one-half to one-quarter that of the larger mode and the latter was usually not an integral multiple of the former. Detailed study was carried out only in diaphragm and soleus muscles from young mature mice, 11–13 months old. In both muscles, the proportion of fibres with bimodal m.e.p.p.s increased to 30 % in low calcium/high magnesium Krebs solution, and dropped to 0 % in cut fibre preparations of soleus and diaphragm (twenty-six and nineteen fibres respectively). In the latter preparations, especially in diaphragm, the decreased resting membrane potential in most cases did not reduce

TABLE 1. Percentage of fibres with bimodal m.e.p.p. amplitude distribution in muscles from young (11–13 month) and old (30–33 month) CBF-1 mice*

	% Bimodal	
Muscle	Young	Old
Extensor digitorum longus	19 % (37)	6 % (35)
Soleus	18 % (33)	23 % (35)
Gluteus maximus	19 % (33)	12 % (34)
Extensor digitorum communis	16 % (37)	19 % (37)
Diaphragm	22 % (28)	21 % (53)

* Number of fibres studied in parentheses.

m.e.p.p. amplitude sufficiently to cause the small mode m.e.p.p.s to be lost in base-line noise. The geometric mean ratio of large to small mode m.e.p.p. amplitude was 2·2 and 2·5 for young and old soleus muscles respectively, and 2·8 and 3·2 for young and old diaphragm (at least eight fibres in each group).

There were two types of bimodal m.e.p.p. amplitude distribution, the more common ('type 1') having smaller modes in which the rise time was about the same as in the larger mode (Figs. 1*B* and 2*D* and *E*; compare to unimodal m.e.p.p., Figs. 1*A* and 2*A* and *B*). Less than 20% of the bimodal distributions fell into another ('type 2')

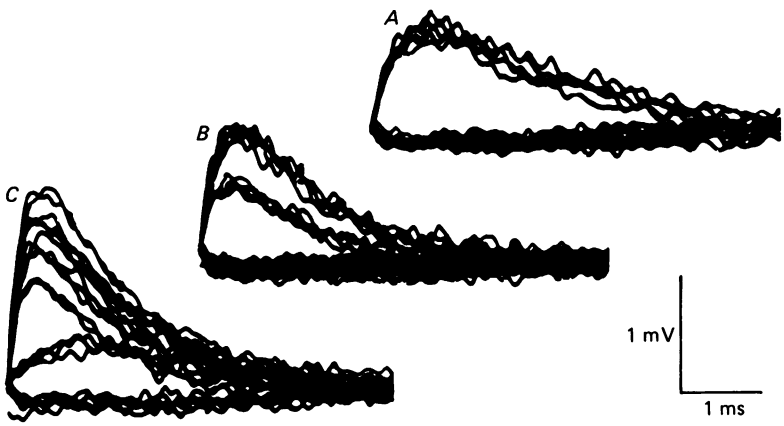

Fig. 1. Superimposed storage oscilloscope traces illustrating unimodal (*A*) or type 1 and 2 bimodal m.e.p.p. distributions (*B* and *C*, respectively). All recordings are to the same scale and are from diaphragm muscle fibres from young (*A* and *C*) or old (*B*) mice.

category in which the rise time of the smaller mode was longer than that of the larger mode (Figs. 1*C* and 2*G* and *H*). In both types of bimodal distribution, half-decay times were longer in the larger mode m.e.p.p.s than in the smaller mode (Fig. 2*F* and *I*). This observation implies that the type 2 small mode m.e.p.p.s do not arise from a distant end-plate on the same fibre (Boyd & Martin, 1956). Also, about twenty to thirty single fibres stained for cholinesterase were examined in each of the muscle types listed in Table 1 (i.e. a total of 188 fibres) and no dual end-plates were found. Comparison of population histograms of modal m.e.p.p. amplitudes in young and old soleus muscle fibres with uni- and bimodal distributions (Fig. 3) indicated that the smaller mode in the bimodal cases formed a separate population, whereas the larger mode was similar to that found in fibres with unimodal m.e.p.p. distribution. A similar comparison in young and old diaphragm fibres yielded the same result.

In the presence of edrophonium (10^{-5} g/ml), there was an increase in amplitude, rise time, and half-decay time in both modes of type 1 distributions (five cases). Because type 2 bimodal m.e.p.p.s were so infrequent, only two cases were observed before and after edrophonium, but in both the smaller mode was no longer present upon exposure to the anticholinesterase.

Effect of potassium. When 1·0 M-potassium chloride was added to the bathing solution, sufficient to raise the bath concentration to approximately 15 mM, the post-synaptic membrane depolarized by $14{\cdot}1 \pm 0{\cdot}7$ mV ($n = 9$) and the over-all

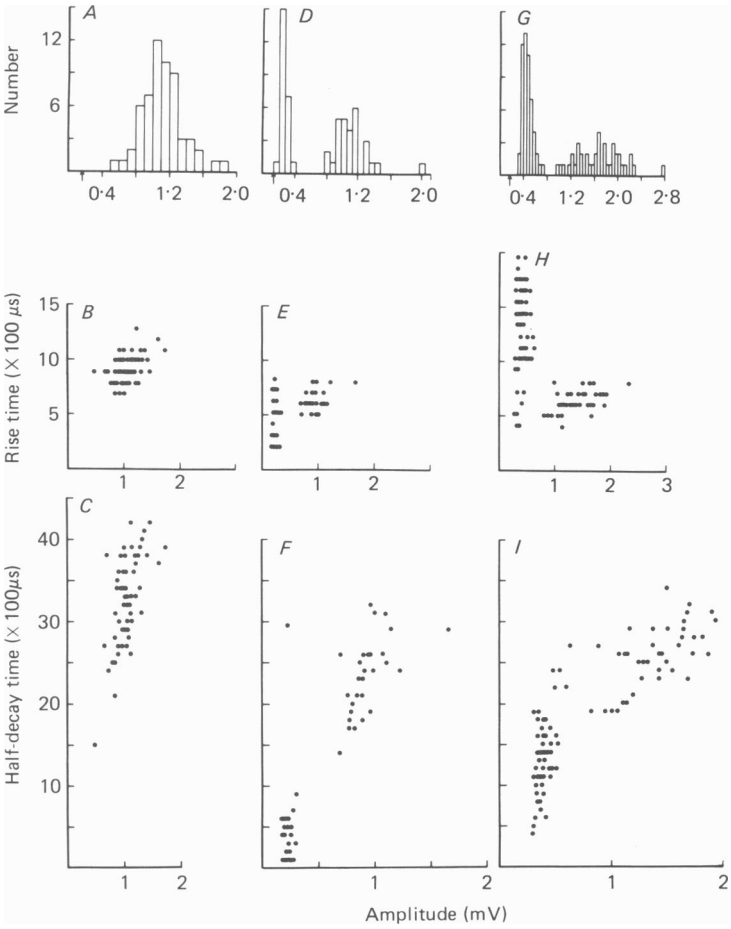

Fig. 2. Examples of single fibre m.e.p.p. amplitude histograms and scatter-plots of rise and half-decay times as a function of amplitude. Results from a fibre with unimodal m.e.p.p.s are given in *A–C*, and from fibres with type 1 and type 2 bimodal m.e.p.p.s in *D–F* and *F–I*, respectively. In *H* and *I*, one or two eccentric data points were omitted for compactness of presentation.

frequency of m.e.p.p.s increased at least 2-fold. In seven fibres with type 1 bimodal distributions, the frequency of the larger mode was always greater. However, the frequency of the smaller mode was increased in only four fibres, decreased in two, and went to zero (no small mode m.e.p.p.s) in one. The smaller mode also disappeared in both type 2 bimodal fibres observed under these conditions.

Effect of calcium/magnesium ratio. When young soleus muscles were placed in low calcium (0·4 mM)/high magnesium (2·75 mM) Krebs solution, there was a reduction in the frequency of m.e.p.p.s in fibres with unimodal distributions. Given the over-all frequency in bimodal distributions, together with the number of m.e.p.p.s in each mode, it was possible to calculate the frequency of each mode before and after exposure to the low calcium solution. The larger mode m.e.p.p. frequency was not

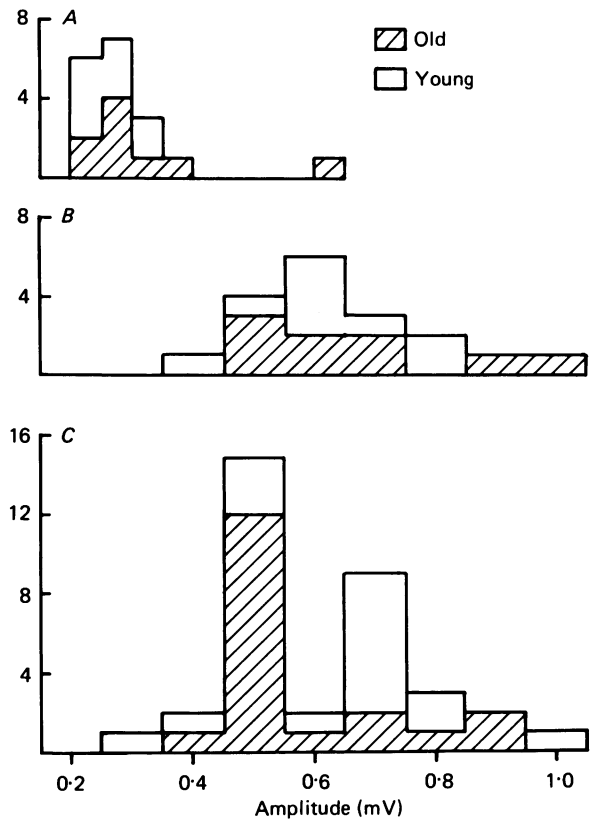

Fig. 3. Histograms showing the distributions of modal m.e.p.p. amplitudes between soleus muscle fibres from young (open bars) and old (hatched bars) mice. The distributions of small modes (*A*) and large modes (*B*) from the same fibres having bimodal m.e.p.p. amplitudes may be compared with that from other fibres having unimodal m.e.p.p. amplitudes (*C*). Data from young mice were added on top of the data from old mice to facilitate the comparison. All these results were obtained from muscles in low calcium/high magnesium solutions.

TABLE 2. Effect of low calcium solution on unimodal and bimodal m.e.p.p. frequencies in young soleus muscles*

	Frequency (Hz)	
	Normal Ca and Mg	Low Ca/high Mg
Unimodal m.e.p.p.	3·21 ± 0·55 (33)*	0·97 ± 0·13 (26)
Over-all bimodal m.e.p.p.	2·15 ± 0·29 (9)	1·33 ± 0·30 (10)
Small mode m.e.p.p.	0·33 ± 0·07 (9)	0·56 ± 0·16 (10)
	0·04 – 0·55	0·08 – 1·48
Large mode m.e.p.p.	1·88 ± 0·26 (9)*	0·77 ± 0·19 (10)
	0·69 – 3·16	0·25 – 2·22

* Values are given as mean ± 1 S.E. of mean, with the number of fibres sampled shown in parentheses. Asterisk indicates significant difference ($P < 0·05$) between frequencies in the two solutions. The range of values is given below the mean value for small and large mode m.e.p.p.s.

significantly different from the unimodal m.e.p.p. frequency and it decreased in low calcium solution by about the same amount as unimodal m.e.p.p. frequency, but the smaller mode frequency was unaffected (Table 2).

Effect of nerve stimulation. M.e.p.p.s and then e.p.p.s were recorded in the same fibres from young soleus and diaphragm muscles after neuromuscular transmission was blocked in low calcium/high magnesium Krebs solution (see Methods). In muscle fibres with bimodal m.e.p.p. amplitudes, the smallest mode e.p.p. was identical in amplitude to the small mode m.e.p.p. (e.g. Fig. 4*A* and *A'*) in seventeen out of eighteen fibres studied, i.e. small mode quanta were evoked by nerve stimulation. In

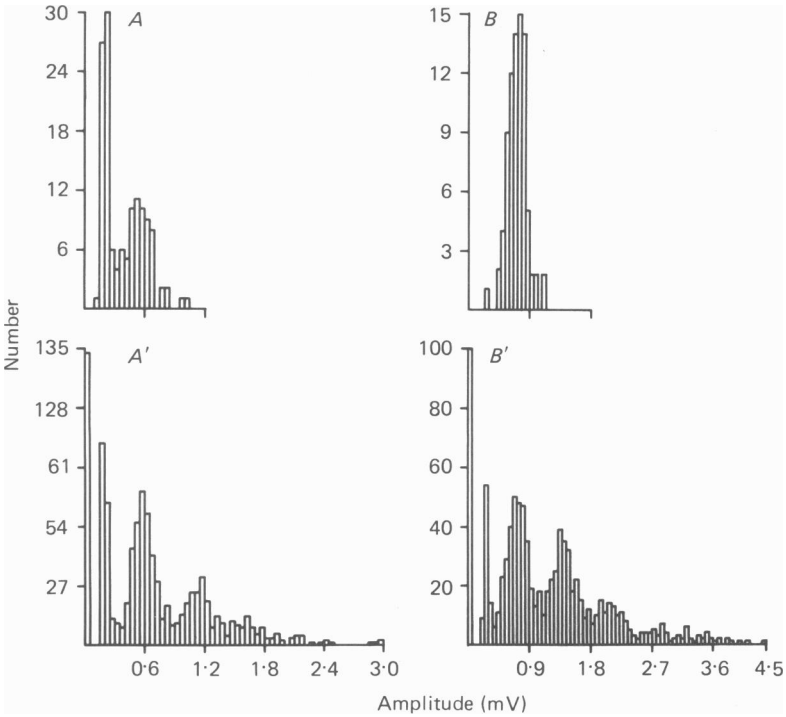

Fig. 4. Amplitude histograms of m.e.p.p.s (*A* and *B*) and corresponding e.p.p.s (*A'* and *B'*) from two soleus muscle fibres (*A* and *A'*, *B* and *B'*). E.p.p.s were evoked in low calcium/high magnesium Krebs solution. In *B*, the spontaneous m.e.p.p. distribution is unimodal but nerve stimulation also evoked a class of small mode e.p.p.s (*B'*) in addition to e.p.p.s corresponding to the unimodal m.e.p.p.

three fibres, trains of 1000 stimuli were used, and the proportion of small mode e.p.p.s produced by serial groups of 50 stimuli was compared throughout the train. No change in the proportion of small mode e.p.p.s was found during the train. Definite peaks corresponding to twice the small mode e.p.p. and not merging into the single unit large mode e.p.p. were observed in only four of the seventeen fibres. In the other thirteen fibres, either there were too few failures of the large mode to permit observation of small mode e.p.p.s, or the smaller values in the large mode distribution overlapped and therefore obscured possible small mode doublets. The quantum content of the small and large mode e.p.p.s was calculated by the method of failures

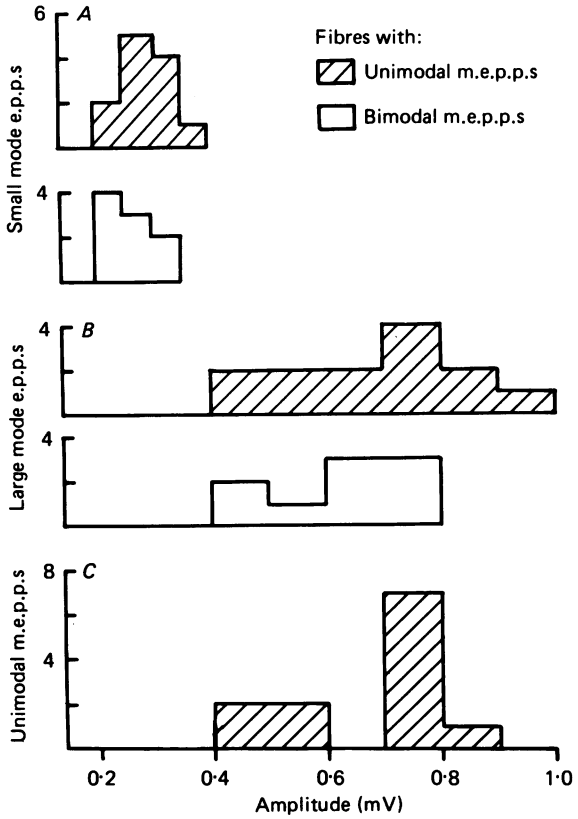

Fig. 5. Comparison of small and large mode e.p.p.s in soleus fibres in which m.e.p.p.s were unimodal (hatched) or bimodal (open boxes). Data are histograms of modal amplitudes, each unit representing a modal value from one fibre. In fibres with unimodal m.e.p.p.s (shown in *C*), e.p.p.s were bimodal (*A* and *B*, cross-hatched) with similar e.p.p. modal values to those in fibres with initially bimodal m.e.p.p.s.

(Del Castillo & Katz, 1954), assuming independent superimposed Poisson distributions of both modes, and counting small mode e.p.p.s (only visible in the absence of large mode e.p.p.s) as failures of the large mode e.p.p. The resulting quantum content was about 0·7 and 1·1 for the small and large mode e.p.p.s, respectively.

Also, twelve out of fifteen soleus muscle fibres which had only unimodal m.e.p.p. distributions before stimulation also showed small mode e.p.p. peaks when the nerve was stimulated (e.g. Fig. 4*B* and *B'*). It was apparent from population histograms of these data (Fig. 5) that the amplitudes of these evoked small mode e.p.p.s were similar to those of the small mode in initially bimodal fibres. The mean e.p.p. amplitude in fibres with small mode m.e.p.p.s (0·51 mV) was not significantly different from the value in fibres with unimodal m.e.p.p.s (0·60 mV). Thus, small mode m.e.p.p.s or e.p.p.s or both were present in over 80% of all randomly selected soleus or diaphragm muscle fibres in low calcium/high magnesium Krebs solution.

DISCUSSION

There were six major findings of this study. First, bimodal m.e.p.p. amplitude distributions were seen in about 20 % of the fibres in several muscle groups from normal adult mice. Secondly, the properties of the larger mode m.e.p.p.s corresponded to those of unimodal m.e.p.p.s. Thirdly, the smaller mode m.e.p.p.s appeared to be focal (short rise time) in the more common (type 1) bimodal distributions. Fourthly, in contrast to unimodal or large mode m.e.p.p.s, the frequency of smaller mode m.e.p.p.s was relatively insensitive to increased potassium or decreased calcium concentrations. Fifthly, the smaller mode potentials could be evoked by nerve stimulation even in muscle fibres which had unimodal spontaneous potentials. Also reported (in a small percentage of fibres) is the existence of a class of bimodal distributions (type 2) in which the small mode shows an unusually long rise and decay time.

As noted in the Introduction (see references there), multimodal m.e.p.p. amplitude distributions have previously been reported at developing, immature or heavily tetanized neuromuscular junctions but not in unselected mature adult animals under unstressed physiological conditions. Our findings of bimodal m.e.p.p. distributions in 20 % of mature mouse fibres may simply reflect the fact that recordings were made from a relatively large number of fibres in each muscle type, that over 200 m.e.p.p.s per fibre were recorded, and that m.e.p.p. histograms were plotted routinely. However, the possibility of a strain-specific finding has not been excluded. The fact that in our unselected muscle fibres, mean amplitude of unimodal m.e.p.p.s coincided with that of the large mode m.e.p.p.s in fibres with bimodal m.e.p.p.s, is counter to the possibility that the 20 % of fibres with bimodal m.e.p.p.s represent a subclass of small diameter fibres, as studied by others (e.g. Bevan, 1976; Kriebel, 1978). If this were the case, the large mode amplitude would be greater than m.e.p.p. amplitude in unimodal fibres. In addition, in the muscles chosen, the standard error of fibre diameter is less than 5 % of the mean (Banker *et al.* 1983).

The increase in the proportion of small mode m.e.p.p.s as external calcium is decreased has also been reported by Kriebel *et al.* (1976), although those authors did not state that this was due to a preferential reduction of frequency of the large mode m.e.p.p.s. In the same vein, the observation of small mode m.e.p.p.s at neuromuscular junctions poisoned with botulinum toxin (Cull-Candy *et al.* 1976) may reflect not the appearance of a new type of m.e.p.p. but the uncovering of an already existing phenomenon by the abolition of the large mode m.e.p.p.s which previously vastly outnumbered the small mode. Indeed, Kriebel *et al.* (1976) were able to eliminate large mode m.e.p.p.s selectively in acute experiments with botulinum toxin, and in our experiments both the amplitude of the small mode m.e.p.p. and the lack of change in frequency with altered external potassium and calcium was similar to that reported after botulinum treatment (Cull-Candy *et al.* 1976).

The small mode m.e.p.p.s in type 1 bimodal distributions have short rise times and therefore cannot originate at a distant end-plate or from Schwann cells (Birks, Katz & Miledi, 1960). Evocation by nerve stimulation also rules out Schwann cells as a possible source. It is also unlikely that type 2 (long rise and decay time) small mode m.e.p.p.s originate at junctions distant from the recording site: first, muscle fibres

stained for cholinesterase never showed two separate end-plates; secondly, half-decay times were shorter than those of large mode m.e.p.p.s at the same junction; thirdly, the effects of edrophonium and potassium in eliminating type 2 small mode m.e.p.p.s were entirely different from those expected of large mode m.e.p.p.s arising from a distant end-plate. Small m.e.p.p.s with long time course, such as these, may arise from release sites located at some distance from the receptors (Kuffler & Yoshikami, 1975). Because of the unusual responses to edrophonium and especially to potassium, it seems more likely that a unique presynaptic terminal release site is involved, such as an ultraterminal sprout. M.e.p.p.s with long rise and decay times were also observed in regenerating junctions (Bennett, Florin & Woog, 1974).

The present results do not allow conclusive evaluation of the hypothesis of Kriebel and co-workers (1976) that large mode m.e.p.p.s and e.p.p.s are made up of multiples of small mode 'subunits'. Our type 1 small mode m.e.p.p.s may not be the same as the 'subunits' reported by Kriebel *et al.* (1976), since their ratios of large to small mode amplitudes of 12–15 in diaphragm muscle at 32 °C differs considerably from our observed ratios of about 3 at 30 °C. Their smaller m.e.p.p.s might have been undetectable in our experiments. In addition, the observation that small mode potentials are produced when the nerve was stimulated in solutions with low calcium/magnesium ratio contrasts with the findings in the frog (Bevan, 1976; but see Kriebel, 1978) and immature mouse (Kriebel, Llados & Matteson, 1982). In addition, Cooke & Quastel (1973) were able to elicit small mode m.e.p.p.s with prolonged nerve terminal depolarization, but during long trains of stimuli at 20 Hz in low calcium/high magnesium solution, we found no change in the proportion of small mode potentials. Thus, in our experiments, evocation of small mode e.p.p.s did not require unphysiological stress. Unfortunately, it was not possible to detect with certainty release of double or multiple small mode quanta, since these often overlapped with small single quanta of the large mode.

There remain several possible explanations for the existence of two populations of m.e.p.p.s at one end-plate (see also discussions in Harris & Miledi, 1971; Dennis & Miledi, 1974; Kriebel & Gross, 1974). Any such explanation would have to take into account not only the different size classes of the m.e.p.p.s but also the new findings reported here, namely the different sensitivities of large and small mode quanta to altered external potassium and calcium and the readily evoked release of small mode quanta in almost all muscle fibres. For example, (i) presynaptic vesicles may have two distinct stages of filling, and partially filled vesicles may combine with release sites differently or combine with a different class of release site; (ii) vesicles may empty in two stages, the first stage being less sensitive to calcium and potassium; (iii) there may be two types of release site, one of which allows only partial release of transmitter; (iv) each vesicle may represent a 'subunit' of release (cf. Kriebel *et al.* 1976) and one subunit may be less dependent on calcium for release than several; or (v) different types of release site and/or vesicles are distributed throughout the nerve terminal or in definite locations, such as on newly formed nerve twigs. Our data do not permit a choice amongst these possibilities. However, the finding that most adult mouse neuromuscular junctions show small mode e.p.p.s implies that small mode transmitter release occurs under physiological conditions. Therefore, an understanding of the origin of these potentials may supply important clues to the mechanism of normal transmitter release.

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