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HISTOGRAMS OF THE UNITARY EVOKED POTENTIAL OF THE MOUSE DIAPHRAGM SHOW MULTIPLE PEAKS

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SUMMARY

1. Two classes of miniature end-plate potentials (m.e.p.p.s) were recorded from diaphragm neuromuscular junctions. Amplitude histograms of both classes had multiple peaks that were integral multiples of the smallest peak (s-m.e.p.p.s). The smaller m.e.p.p.s formed the first three or four peaks of histograms and the number of m.e.p.p.s (skew-m.e.p.p.s) in each peak decreased, forming an over-all skewed distribution. The larger m.e.p.p.s (bell-m.e.p.p.s) formed a more-or-less bell-shaped distribution. The distribution of m.e.p.p.s varied from mainly skew- to mainly bell-m.e.p.p.s. In young adult mice the number of subunits composing the classical m.e.p.p.s varied between ten and fifteen at room temperature; at higher temperatures the range was from three to ten subunits.

2. End-plate potentials (e.p.p.s) were reduced with cobalt ions (*ca.* 4 mM) until most nerve impulses failed to release transmitter. The amplitudes of 'unitary evoked potentials' were of the bell-m.e.p.p. class and histograms show integral multiple peaks that correspond to the peaks in histograms of the bell-m.e.p.p.s.

3. The peaks in both m.e.p.p. and unitary e.p.p. histograms remained in the same position throughout the recording period and became more distinct as the sample size increased.

4. The variance of the s-m.e.p.p. was estimated from the noise and measurement error and the variance of all peaks in the histograms. Most variance of the first peak (s-m.e.p.p.) was due to noise and measurement error.

5. The integral peaks in the m.e.p.p. and 'unitary evoked potential' histograms are predicted with a probability density model based on the estimated variance of the s-m.e.p.p. and the assumption that larger potentials are composed of subunits the size of s-m.e.p.p.s. The data and model support the hypothesis that m.e.p.p.s and unitary potentials are composed of subunits.

INTRODUCTION

By using small muscle fibres of the frog sartorius which generated relatively large miniature end-plate potentials (m.e.p.p.s) Kriebel & Gross (1974) showed that there were two distinct classes of miniature end-plate potentials originating at the same

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junction. The smaller m.e.p.p.s formed a distinct peak (s-m.e.p.p.s) and appeared similar to those reported by Cooke & Quastel (1973) from the rat diaphragm. S-m.e.p.p.s also formed the predominant class early in the developing tadpole leg muscle (Kriebel & Gross, 1974) indicating that they may represent the same class of skew m.e.p.p.s reported by Dennis & Miledi (1971, 1974) at the regenerating neuromuscular junction (see Muniak, 1980, for mouse neuromuscular junction). Kriebel & Gross (1974) found m.e.p.p. amplitude histograms of normal adults with four or five peaks that were integral multiples of the first peak and they noticed that the number of peaks, but not peak intervals, was readily changed with various challenges (heat and nerve stimulation). They suggested that the multiple peaks in m.e.p.p. histograms resulted from the summation of subunits. Further evidence for the subunit hypothesis was presented by Kriebel (1978) who showed that multiple exposures of m.e.p.p.s which triggered the oscilloscope exhibited preferred m.e.p.p. amplitudes that corresponded to the integral peaks in histograms composed of the same m.e.p.p.s. Wernig & Stirner (1977) also found that frog m.e.p.p. amplitude histograms showed integral multiple peaks and these were sharpened when m.e.p.p.s generated from a restricted part of the junction were used to construct histograms (Wernig & Motelica-Heino, 1978). On the other hand, Miller, Weinstock & Magleby (1978) ascribed the peaks in their histograms to random variation in small sample sizes ('random variation' hypothesis). However, Kriebel & Gross (1974), and Kriebel, Llados & Matteson (1976) demonstrated stationarity of peaks in successive periods and they were able to change the mean m.e.p.p. amplitude with various challenges (such as temperature, colchicine, botulinum toxin and nerve stimulation) without altering the peak interval. Moreover, Wernig & Motelica-Heino (1978) and Carlson (1980) increased m.e.p.p. amplitudes with neostigmine and found that the peak intervals increased by the same percentage as the mean m.e.p.p. These observations support the subunit hypothesis.

There have been two major problems with the subunit hypothesis. The first concerned the availability of the subunit for evoked release. Bevan (1976) showed that in the unstressed frog preparation, e.p.p.s the size of smaller, skewed m.e.p.p.s were not evoked. On the other hand, Kriebel (1978) found that in some unstressed preparations and in all preparations after intense periods of nerve stimulation e.p.p.s the size of s-m.e.p.p.s were evoked. We demonstrate here the generation of evoked potentials the size of s-m.e.p.p.s in the mouse preparation. A second problem concerned the sharp peaks in m.e.p.p. amplitude distributions. Bevan (1976), Katz (1977), and Miller *et al.* 1978, have pointed out that the variance of larger peaks did not increase rapidly enough to result from added subunits that have the variance of the first peak. However, the variance of the first peak (s-m.e.p.p.s) should not be used because it represents not only the actual variance of the s-m.e.p.p. but a variance component due to recording noise and measurement error. We have shown that the peaks in m.e.p.p. amplitude histograms are expected with a subunit model which uses the predicted variance of the subunit (Matteson, Kriebel & Llados, 1979). We report here that histograms of 'unitary evoked' potentials show integral peaks and that these peaks correspond to those in m.e.p.p. histograms and that these histograms can be fitted with a subnunit model.

METHODS

Preparation and recording

Hemi-diaphragms with phrenic nerves of young mice (usually 2–3 weeks) were removed and placed in a constant temperature bath mounted onto the stage of a compound microscope. Buffered saline (pH 7·4, bicarbonate-phosphate buffer) was recirculated with a bubble lift (95 % O_2, 5 % CO_2) and maintained within 1 °C at the desired experimental temperature (22–38 °C). The electrophysiological methods were similar to those reported in Kriebel *et al.* (1976). The phrenic nerve was drawn into a suction electrode against an internal Ag-AgCl wire to ensure electrical contact. Junctions that appeared to show multiple innervations were not used (Bennett & Pettigrew, 1974). Transmitter release was reduced with Co ions, adjusted (around 4 mM) so that most nerve action potentials failed to release transmitter. Since m.e.p.p.s and e.p.p.s were quite large and near threshold, we usually did not use neostigmine. In most experiments, our noise level was under 100 μV peak-to-peak. Experiments were recorded on magnetic tape and later filmed. The camera speed was 10 or 20 mm/sec and the oscilloscope sweep was usually 20 msec/cm. This produced a slanted base line. Film negatives were enlarged and projected onto graph paper so that the oscilloscope trace was contained within two lines of the graph paper. The peaks of m.e.p.p.s were easily seen and the amplitudes readily measured by placing a line on the graph paper over the oscilloscope trace. Potential amplitudes were measured to an accuracy of half the distance between lines on the graph paper. The film was developed so that m.e.p.p. peaks near preceding oscilloscope traces could be determined to within the reading error. The e.p.p.s were usually read from a second film produced from the magnetic tape. The oscilloscope trace was triggered with the negative stimulus artifact and the sweep rate (2 msec/cm) was great enough so that the evoked potential was spread across the entire trace. There was no difficulty in determining the evoked peak or amplitude. The histograms of evoked potentials produced with both triggered and free-running sweeps were the same. The histograms presented here show distinct integral peaks composed of 5–7 histobars. The film was read in four to ten serial segments to check for stationarity of peaks and mean amplitudes. Amplitudes were either written down or recorded by a second person. As a further check against a measuring bias, the film was enlarged further by an arbitrary amount and read by a second person. After the data were measured, the enlargement was determined and the m.e.p.p. and e.p.p. distributions measured by the two readers compared. The average potential and the peak intervals were increased by the same percentage at the larger enlargement. We found the same number of peaks at both film enlargements and no obvious reading biases between different readers.

Calibration pulses were used to determine the variance of m.e.p.p.s (s-m.e.p.p.s) due to noise and reading error. A relatively long calibration pulse (20 msec) showed no detectable variance when measured from average base line to average pulse height. This procedure cannot be utilized in our study because a m.e.p.p. (or e.p.p.) has a peak with a shape corresponding to the most common noise (*ca.* 60 Hz) frequency. Therefore, we measured a short calibration pulse to determine the noise component (σ^2_m, see model below) of measured m.e.p.p.s.

Model

We have derived a model of m.e.p.p. and e.p.p. amplitude distributions, based on the subunit hypothesis, in order to test the significance of integral multiple peaks in amplitude histograms. The following three assumptions were used to derive the model, which is simply a probability density function of m.e.p.p. amplitudes.

(1) S-m.e.p.p.s result from the release of a subunit of transmitter. The subunit amplitude is assumed to be normally distributed with mean (μ) and variance ($\sigma^2 + \sigma^2_m$), where (σ^2) is the actual variance of the subunit response and (σ^2_m) represents measurement and noise error.

(2) Larger amplitude m.e.p.p.s result from the summation of two or more subunits. Subunits are assumed to sum linearly and independently; therefore, the release of j subunits ($j = 1, 2, 3 \ldots$) would produce a normally distributed subpopulation of m.e.p.p. amplitudes with mean ($j\mu$) and variance ($j\sigma^2 + \sigma^2_m$).

(3) The over-all amplitude distribution is, therefore, composed of a mixture of these subpopulations. Each subpopulation must be muliplied by a weighing factor (Wj) which represents the probability that a m.e.p.p. belongs to the jth subpopulation. Each Wj, therefore, determines the relative contribution of the jth subpopulation to the overall distribution. The probability density function of k subpopulations is, therefore, as follows:

$$f(x) = W_1 \cdot N_1(x) + W_2 \cdot N_2(x) + \ldots + W_k \cdot N_k(x),$$

where f(x) represents the probability density of observing a m.e.p.p. of amplitude x and $N_j(x)$ is the normal probability density function with mean ($j\mu$) and variance ($j\sigma^2 + \sigma^2_m$).

For any observed amplitude histogram, estimates of the parameters in this function (W_1, W_2, ..., W_k, μ and σ) were obtained as maximum likelihood estimates (Mood, Graybill & Boes, 1974). The estimation procedure involves finding the values of the parameters that simultaneously maximize the likelihood function. In other words, all the data of a given histogram are utilized in estimating these parameters. For example, the estimated subunit variance (σ^2) was computed from the experimentally determined measurement and noise variance (σ^2_m) and the variances of all observed peaks. Note that σ^2_m is not a parameter of the model. This constant was evaluated, for each end-plate studied, by calculating the variance of the amplitude of a series of calibration pulses from a Bioelectric CA5 calibrator. Once the estimates of the parameters were obtained the probability density function was used to produce a fit to the observed histogram. These fits are illustrated by the continuous curves in the Figures published here.

The significance of the fit produced by the above multimodal model was tested in two ways. First of all, the model was tested against a reduced, bimodal model using a generalized likelihood ratio test for large sample sizes (Mood *et al.* 1974). This test basically involves calculating a χ^2 (which is abbreviated χ^2_{LRT} in the text) which, if significant, indicates that the multimodal model fits the data significantly better than the bimodal model. Secondly, as an indication of the accuracy of the multimodal model, we tested observed m.e.p.p. and e.p.p. amplitude histograms against this model using a χ^2 goodness of fit test (Steel & Torrie, 1960). These χ^2 statistics are abbreviated χ^2_{GFT} in the text. A significance level of 0·05 was chosen for these tests. See Matteson (1979) and Matteson *et al.* (1979) for a detailed description of this analysis as applied to m.e.p.p.s.

RESULTS

M.e.p.p. and e.p.p. amplitude histograms at room temperature

Most of our experiments were performed at room temperature (20–25 °C) or 30–32 °C because the preparations did not shift position against the restraining hooks and the muscle fibre membranes appeared to seal around the electrode tip better than at 35 °C (mouse temperature). Also, at room temperature, the m.e.p.p.s were larger than at 35 °C. We added an anticholinesterase agent at 30 °C and found no increase in m.e.p.p. amplitude suggesting that the cholinesterase was not active below 30 °C.

At room temperature, the most striking aspect of m.e.p.p. histograms was the large number of smaller m.e.p.p.s that comprised 10–20% of the m.e.p.p.s (*ca.* 100 preparations) (Fig. 1*A*) (see Kriebel *et al.* 1976). The over-all m.e.p.p. amplitude profile can be divided into two parts. The smaller m.e.p.p.s form an overall skewed distribution (skew-m.e.p.p.s) and the larger m.e.p.p.s form an over-all bell-shaped distribution (bell-m.e.p.p.s) (Matteson *et al.* 1979). The bell-m.e.p.p.s correspond to the classical m.e.p.p.s (Liley, 1956*a*; Boyd & Martin, 1956*a*).

The second aspect of the m.e.p.p. distribution is that both the skew- and bell-m.e.p.p.s exhibit integral multiple peaks. The multiple peaks were observed in every preparation and muscle fibre in which we had a large signal-to-noise ratio, a stable resting potential and a relatively low m.e.p.p. frequency. Sometimes the middle integral peaks were apparent after reading 100–200 m.e.p.p.s and additional peaks became apparent with greater m.e.p.p. numbers. In other preparations, 200 m.e.p.p.s were not an adequate sample size and 600–1000 m.e.p.p.s were required to demonstrate integral peaks. Once peaks became apparent, they remained stationary (see Kriebel *et al.* 1976; Matteson *et al.* 1979; Llados, *et al.* 1980). These observations rule out the possibility that the peaks result from chance variations of sample size, since peaks which result from variations in a Gaussian distribution become less prominent, and

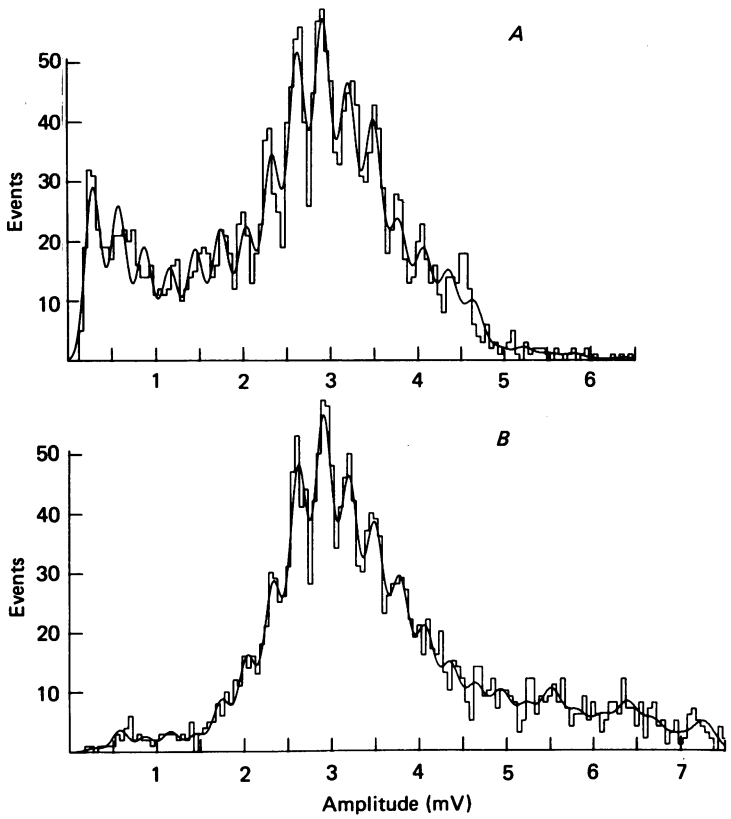

Fig. 1. M.e.p.p. and e.p.p. amplitude distributions from a 14 day old mouse diaphragm junction at room temperature. 25 °C. No neostigmine. The phrenic nerve was stimulated at 2 Hz and all m.e.p.p.s and e.p.p.s were measured during the 70 min of continuous recording. The continuous lines show the predicted distributions based on the subunit hypothesis. The noise and measurement standard deviation (σ_m) was 0·09 mV. Membrane potential was −64 mV throughout, Co^{2+} was *ca.* 4 mM. *A*, m.e.p.p.s. Note that there are two general classes of m.e.p.p.s. The smaller ones form a skew distribution and, in this case, show two distinct peaks. $2{\cdot}4 \times 10^3$ m.e.p.p.s (34/min) compose this histogram of which 20 % are skew-m.e.p.p.s and 5 % are s-m.e.p.p.s. The estimated value of subunit size (μ) was 0·29 mV and of subunit standard deviation (σ) was 0·016 mV. $\chi^2_{LRT} = 155{\cdot}7$ with 18 d.f.; $P < 0{\cdot}001$. $\chi^2_{GFT} = 59{\cdot}74$ with 54 d.f.; $P > 0{\cdot}2$. *B*, e.p.p.s. Since most stimuli resulted in failures (75 %), most e.p.p.s correspond to the classical unitary evoked potential. The few larger e.p.p.s (6 mV) represent two classical unitary evoked potentials. Note that few e.p.p.s the size of s-m.e.p.p.s or skew-m.e.p.p.s are present. The e.p.p. histogram is not smooth but shows multiple peaks which are in register with those in the m.e.p.p. amplitude distribution. There are $2{\cdot}5 \times 10^3$ e.p.p.s in this histogram. $\mu = 0{\cdot}29$ mV and $\sigma = 0{\cdot}018$ mV. $\chi^2_{LRT} = 558{\cdot}8$ with 21 d.f.; $P < 0{\cdot}001$. $\chi^2_{GFT} = 46{\cdot}7$ with 71 d.f.; $P > 0{\cdot}98$.

change position, as the sample size increases (Miller *et al.* 1978). Experimentally, it was sometimes possible to record 3000–5000 m.e.p.p.s in 1–2 hr with little (1–3 mV) or no membrane potential change. Fig. 1 is representative of fibres from twenty preparations in that the bell-m.e.p.p.s show several integral peaks and in this example the intervals are 5·75 histobars (the estimated mean of the s-m.e.p.p.s, μ, was 290 μV).

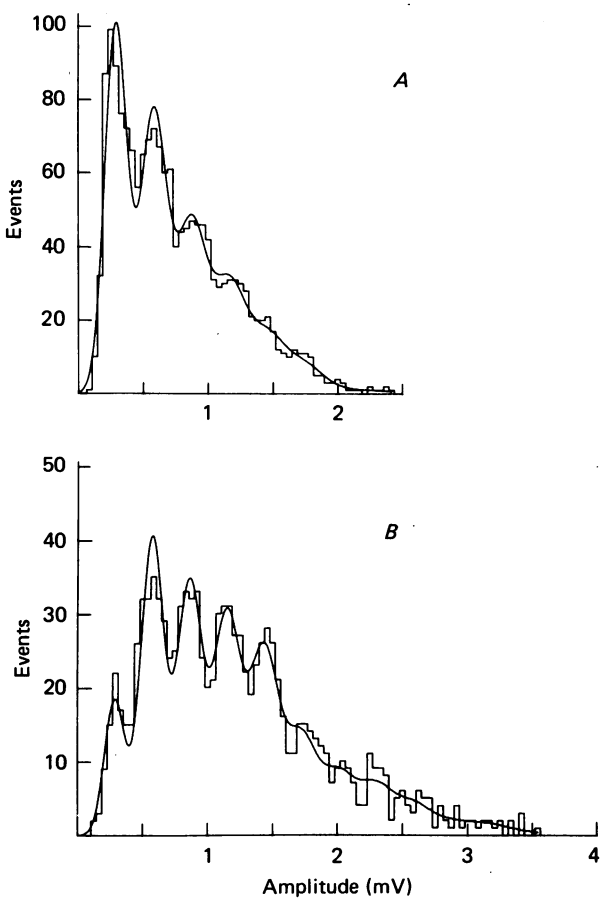

Fig. 2. M.e.p.p. and e.p.p. amplitude distributions from a 15-day-old mouse diaphragm at 35 °C. An extreme example where the m.e.p.p.s are mainly of the skew-class. Neostigmine, 10^{-6} g/ml. The phrenic nerve was stimulated at 2Hz and all m.e.p.p.s and e.p.p.s generated during the 25 min of continuous recording are shown. The continuous lines show the predicted distributions based on the subunit hypothesis. The standard deviation of the noise and measurement (σ_m) is 0·067 mV. Membrane potential was −62 mV throughout, Co^{2+} was *ca.* 4 mM. *A*, m.e.p.p.s. The bell-m.e.p.p.s do not form a class distinct from the skew-m.e.p.p.s because their frequency was too low. The first four peaks were stationary throughout the recording period. The peak intervals are 7·0 histobars. There are $1{\cdot}8 \times 10^3$ m.e.p.p.s in this histogram (72 m.e.p.p.s/min). μ was 0·29 mV and σ was 0·053 mV. $\chi^2_{LRT} = 177{\cdot}7$ with 4 d.f.; $P < 0{\cdot}001$. $\chi^2_{GFT} = 26{\cdot}9$ with 26 d.f.; $P > 0{\cdot}3$. *B*, e.p.p.s. The high frequency of failure of release (80 %) indicates that most e.p.p.s are classical unitary evoked potentials. Note that the over-all distribution is greatly different from that of the m.e.p.p.s in that the over-all e.p.p. distribution is bell-shaped. However, there are peaks that closely match the peak intervals in the m.e.p.p. distribution (μ was 0·29 mV and σ was 0·040 mV). Small e.p.p.s the size of s-m.e.p.p.s or skew-m.e.p.p.s were evoked but the numbers more or less fit those expected from an over-all bell-shaped distribution. There are $1{\cdot}2 \times 10^3$ e.p.p.s in this distribution. The few larger e.p.p.s forming a right hand skew would result from two or three classical unitary evoked potentials. However, there were not enough larger potentials to show peaks comparable to those reported by Boyd & Martin, 1956*b*, and Liley, 1956*b*. $\chi^2_{LRT} = 248{\cdot}1$ with 8 d.f.; $P < 0{\cdot}001$. $\chi^2_{GFT} = 19{\cdot}2$ with 37 d.f.; $P > 0{\cdot}98$.

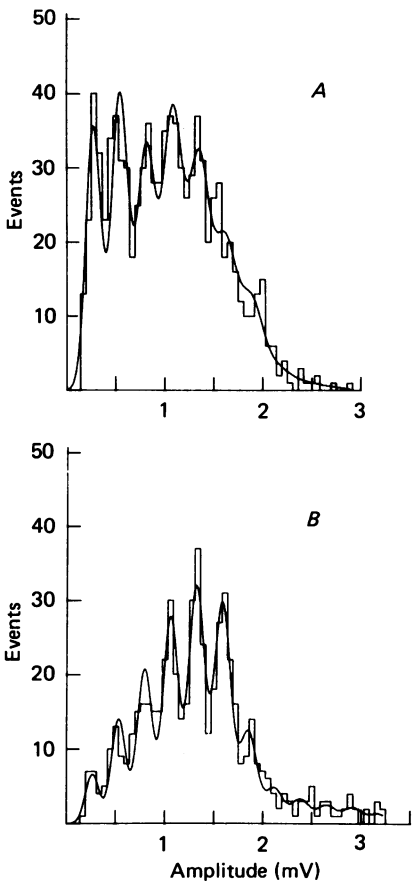

Fig. 3. M.e.p.p. and e.p.p. amplitude distributions from a 13-day-old mouse. Temperature 35 °C, neostigmine, 10^{-6} g/ml. The continuous lines show the predicted distribution based on the subunit hypothesis. The standard deviation of the noise and measurement error (σ_m) is 0·067 mV. The resting potential dropped from −67 to −65 mV for the 35 min of recording time, Co^{2+} *ca.* 4 mM. *A*, m.e.p.p.s. This m.e.p.p. distribution was chosen because the ratio of skew- to bell-m.e.p.p.s was about one. The peak intervals are 4·75 histobars. $9{\cdot}0 \times 10^2$ m.e.p.p.s are in this histogram (24 m.e.p.p.s/min). μ was 0·27 mV and σ was 0·037 mV. $\chi^2_{LRT} = 40{\cdot}9$ with 6 d.f.; $P < 0{\cdot}001$. $\chi^2_{GFT} = 25{\cdot}2$ with 22 d.f.; $P > 0{\cdot}2$. *B*, e.p.p.s 80 % evoked failure rate (e.p.p. reduced with Co^{2+}) indicates that most e.p.p.s were the classical unitary evoked potentials. Peak intervals are the same as in the m.e.p.p. histogram ($\mu = 0{\cdot}26$ mV and $\sigma = 0{\cdot}021$ mV). Note that the over-all shape is more or less bell-shaped and the number of evoked potentials the size of s-m.e.p.p.s fits a bell-shaped envelope. $5{\cdot}0 \times 10^2$ e.p.p.s are in this histogram. $\chi^2_{LRT} = 103{\cdot}7$ with 8 d.f.; $P < 0{\cdot}001$. $\chi^2_{GFT} = 8{\cdot}87$ with 24 d.f.; $P > 0{\cdot}9$.

The same m.e.p.p.s were read at a greater film enlargement and the same number of discrete peaks were found and the average m.e.p.p. amplitude and peak interval were both increased by *ca.* 25 %. The largest peak height is 10 times that of the smallest peak and all larger peaks are an integral multiple of the smallest peak. The positions (mV) of these peaks were stationary throughout the 70 min of continuous recording during which time the phrenic nerve was stimulated at 2 Hz. Cobalt ions had been

added (*ca.* 4 mM) until most nerve stimuli resulted in failures (75 %) so that most e.p.p.s represent the classical unitary evoked potential (Boyd & Martin, 1956*b*; Liley, 1956*b*) with mean amplitude comparable to the mean of the bell m.e.p.p.s (Fig. 1*B*). Note that very few e.p.p.s with skew-m.e.p.p. amplitudes are present and that the amplitude distribution of unitary evoked potentials shows multiple peaks also with intervals of 5·75 histobars (the enlargement gave intervals of 7·20 histobars). Moreover, the e.p.p. peaks are superimposable on the m.e.p.p. peaks. The larger e.p.p.s forming the right hand skew (*ca.* 6 mV) probably represent two summed unitary evoked responses.

M.e.p.p. and unitary evoked potential amplitude histograms at mouse temperature (35 °C)

At 35 °C the over-all profile of m.e.p.p. histograms was extremely variable. We found m.e.p.p. distributions varied within preparations. Moreover m.e.p.p. amplitude profile and the skew- to bell-m.e.p.p. ratio was very sensitive to slight variations in temperature (see Kriebel *et al.* 1976 for examples). Sometimes distributions showed a large percentage of skew-m.e.p.p.s as in Fig. 1*A*. In other preparations only the bell m.e.p.p.s were present and the mean amplitude was 5–8 times that of the first peak; whereas at room temperature, the mean m.e.p.p. amplitude was 10–15 times that of the first peak. The m.e.p.p. distribution in Fig. 2*A* shows an extreme distribution in that the bell m.e.p.p. class is not evident and most m.e.p.p.s are skew-m.e.p.p.s. These distributions were common and we saw them in over thirty preparations. In the preparation shown in Fig. 2 the cobalt concentration was adjusted so that 80 % of the nerve terminal action potentials failed to elicit transmitter release. This high percentage of failures implied that almost all e.p.p.s were unitary. Fig. 2*B* shows that the unitary e.p.p. histogram is composed of multiple peaks. Also note that the unitary evoked potential is substantially larger than the m.e.p.p. and the over-all e.p.p. profile is bell-shaped and not skewed as the m.e.p.p. distribution (Fig. 2*A*).

Fig. 3*A* was chosen to show a skew- to bell-m.e.p.p. ratio near unity and to represent a middle condition between the m.e.p.p. distributions shown in Figs. 1*A* and 2*A*. In Fig. 3*A* the over-all profile has a flat top because the bell-m.e.p.p. mean is only 4 times that of the s-m.e.p.p. and the percentage of skew-m.e.p.p.s is high. In this example, the bell-m.e.p.p.s are not masked by the skew-m.e.p.p.s as shown in Fig. 2*A*. The over-all e.p.p. distribution of unitary evoked potentials is bell-shaped and shows clear integral peaks that match those of the m.e.p.p.s. Moreover, the e.p.p. mode is at the 4th and 5th peak and matches those of the bell-m.e.p.p.s (Fig. 3*A*). These distributions were common but due to the difficulty in obtaining proper signal-to-noise ratios to clearly define the s-m.e.p.p. peak we were not able to determine the percentage of junctions with m.e.p.p. amplitude distributions like those shown in Figs. 2 and 3.

DISCUSSION

By recording from small muscle fibres of young mice, which generate relatively large m.e.p.p.s and by using relatively low resistance micropipettes, we have shown that m.e.p.p. distributions are not always bell-shaped. Distributions skewed into the noise, were uniform, exhibited skew-m.e.p.p.s and bell-m.e.p.p.s or were composed of essentially bell-m.e.p.p.s. The range of distributions has been found in young and in

young adult mice. Adults usually exhibit mainly bell distributions (Carlson, 1980). Moreover, m.e.p.p. and e.p.p. distributions show the same integral peaks even though the over-all amplitude profiles of m.e.p.p.s and unitary e.p.p.s may greatly differ (Figs. 2 and 3). Earlier workers studied m.e.p.p.s of 0·3–0·4 mV at noise levels of 100 μV or more, which may explain the absence of peaks in earlier investigations. We found that peaks remained in the same position for long periods of time (up to 4 hr) providing there was no change in the resting membrane potential (also see Kriebel *et al.* 1976; Matteson *et al.* 1979). Miller *et al.* (1978) found peaks in small sample sizes of randomly selected m.e.p.p.s of a Gaussian distribution and found that the peak intervals were not integral. Moreover, the peaks changed position and became less distinct as the sample size increased from 300 to 1800 m.e.p.p.s. Since our peaks became sharper as the sample size increased (up to 5,000 m.e.p.p.s) the peaks in the histograms shown here are not due to small sample sizes from a Gaussian distribution. In addition, Carlson (1980) has shown that an anticholinesterase increased the average m.e.p.p. and the peak interval by the same percentage. One final observation that is important for the non-Gaussian distribution shown in the histograms presented here is that the m.e.p.p. and unitary evoked potentials both show multiple peaks that have the same peak interval. At room temperature, m.e.p.p. amplitude histograms showed an obvious skew class which composed about 20% of the total m.e.p.p.s. The skew distribution also showed multiple peaks and when present, were found to have the same interval as those peaks in the bell-shaped class (Matteson *et al.* 1979). This observation suggests that both skew- and bell-m.e.p.p.s are composed of the same subunit. Cooke & Quastel (1973) observed the skew-m.e.p.p.s after tetanic stimulation and Cull-Candy, Lundh & Thesleff (1976) observed them in normal rat preparations at 37 °C.

Few e.p.p.s the size of skew-e.p.p.s were evoked with nerve stimulation. Bevan (1976) also found that small e.p.p.s the size of the sub-m.e.p.p. were not evoked in the frog preparation. However, Kriebel (1978) found that after periods of tetanic nerve stimulation, small e.p.p.s were evoked and these were the same amplitude as the s-m.e.p.p. Cull-Candy *et al.* (1976) found that chronic botulinum toxin (BTX) poisoning produced mainly small m.e.p.p.s and these appear identical to those reported by Kriebel *et al.* (1976) (following acute BTX poisoning) which remained after the bell-m.e.p.p.s were blocked. Cull-Candy *et al.* (1976) found that their small skew-m.e.p.p.s were initially evoked and prolonged tetanic stimulation changed the m.e.p.p. and unitary evoked potential to the large classically sized m.e.p.p.s and unitary e.p.p.s respectively. Matteson (1979) found that the smaller potentials were not evoked with acute BTX poisoning although the distribution of m.e.p.p.s was changed to the bell-m.e.p.p. class and unitary e.p.p.s of similar size were evoked during nerve stimulation. These studies show that the ratio of skew- to bell-m.e.p.p.s can be altered with various challenges.

We have shown that the amplitude distributions of m.e.p.p.s and unitary e.p.p.s show integral multiple peaks and that these peaks can be explained with a hypothesis that assumes that the first peak represents the action of a subunit and larger peaks represent the summed action of two or more subunits. We have described a subunit model which fits the rather sharp peaks by demonstrating that most of the observed variance of the first peak does not reflect the intrinsic variance of the subunit but

results from measurement error and noise in the recording system. Since this variance would be constant for each peak in the histogram, the variance of larger amplitude peaks would not be some integral multiple of the measured s-m.e.p.p. variance but mainly of the intrinsic subunit variance. Boyd & Martin (1956*b*) also found that the multiple peaks of e.p.p.s were sharper than predicted on the apparent variance of the unitary evoked potential and they noted that the noise fluctuation added to the scatter of the spontaneous potentials.

It is important to point out that not all m.e.p.p. nor unitary e.p.p. amplitude distributions showed clear peaks as shown in Figs. 1, 2 and 3. We have noted that a sudden decrease in resting potential of a few millivolts to a new steady membrane potential changed the peak interval 5–8 %. Consequently, a gradual drift in membrane potential of only a few millivolts was sufficient to mask the integral multiple peaks. For example, the peak intervals of 290 μV in the bell-shaped part of Fig. 1*A* would be obliterated if the m.e.p.p. amplitudes decreased by 5 %, which would give a subunit size of 275 μV. This change in subunit amplitude would not be detected, although the major peak (composed of ten subunits) would be reduced to 2·75 mV and would fill in the trough between the 9th and 10th peaks in Fig. 1*A*.

The extremely wide range of m.e.p.p. amplitude profiles has not been previously studied in comparison to e.p.p. distributions. Kriebel *et al.* (1976) reported that profiles of m.e.p.p. amplitudes were variable and that the percentage of skew-m.e.p.p.s to bell-m.e.p.p.s was readily altered with various challenges. In this report we have selected three very different m.e.p.p. amplitude profiles and compared them to e.p.p. profiles. In some cases, the bell-m.e.p.p.s dominated the histogram (Fig. 1*A*) and this distribution was usually seen at room temperature. At mouse temperatures (35 °C), the skew-m.e.p.p.s sometimes dominated the distribution such that the bell-m.e.p.p.-class was masked (Fig 2*A*) although the unitary e.p.p.s formed a normal distribution. Cull-Candy *et al.* (1976) observed similar distributions with chronic BTX poisoning in that most m.e.p.p.s were small and probably of the skew class (cf. Kriebel *et al.* 1976, for *in vitro* BTX studies) and that the unitary-e.p.p.s were larger.

End-plate potentials the size of the skew-m.e.p.p.s did not form a distinct class at room temperature. However, unitary e.p.p.s the size of s-m.e.p.p.s were evoked when the bell-m.e.p.p. distribution included s-m.e.p.p.s (Figs. 2*B* and 3*B*). The range of m.e.p.p. distributions was from almost all bell-m.e.p.p.s (Fig. 1*A*) to almost all skew-m.e.p.p.s (Fig. 2*A*). The m.e.p.p. distribution in Fig. 3*A* is between the extremes in that the proportion of bell- to skew-m.e.p.p.s is about unity. The unitary e.p.p.s of Fig. 3*B*, show a bell distribution. It was common that the unitary e.p.p.-distribution was much larger than the m.e.p.p. distribution (Figs. 2 and 3). In examples that showed multiple peaks, the peak intervals were the same in m.e.p.p. and unitary evoked e.p.p. distributions regardless of the m.e.p.p. distribution. This result supports the subunit hypothesis.

A morphological correlate for the subunit cannot be proposed at this time. There is no supportive evidence that a subunit (s-m.e.p.p.) results from exocytosis of one synaptic vesicle. Heuser *et al.* (1979) with freeze-fracture studies found no evidence that rows of vesicles were discharged together. Katz & Miledi (1979) increased the quantal content with diaminopyridine so that more quanta were released than the number of vesicles in contact with the terminal membrane at the 'release-sites'. These

observations favour the hypothesis that one vesicle represents one quantum. It is possible to speculate that the subunits of the m.e.p.p. and unitary evoked potential are contained within or on the vesicular membrane.

In summary, the observations that m.e.p.p. and unitary e.p.p. distributions can be very different but show multiple peaks with the same intervals are evidence that both are composed of subunits.

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