

# Multimodal Distribution of Frog Miniature Endplate Potentials in Adult, Denervated, and Tadpole Leg Muscle

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**ABSTRACT** Amplitude histograms of spontaneous miniature endplate potentials (MEPPs) from adult sartorius muscle cells show a definite bimodality with the mean amplitude of the larger mode five to seven times that of the smaller mode which accounted for 2-5% of the total MEPPs. Histograms were plotted after high frequency MEPP generation induced by increasing temperature, increasing external calcium or nerve stimulation. These plots showed a reversible left-shift of the major mode as well as a reversible increase in the proportion of small mode MEPPs. Repeated challenges shifted almost all MEPPs into the small mode. An increase in the percentage of small mode MEPPs also occurred spontaneously during the course of denervation before the quiescent period and some of the histogram profiles showed multiple modes whose means were integer multiples of the small mode mean. In the early stages of hind leg development the greatest proportion of MEPPs were of the small mode size; as metamorphosis progressed, the histograms showed a definite multimodality with the mean of each mode being an integer multiple of the small mode mean and with the proportion of MEPPs in each mode about the same. During tail resorption the percentage of larger MEPPs increased until the adult histogram profile was reached. Thus, the changes in MEPP amplitude histograms over the course of metamorphosis are the reverse of those found with denervation.

## INTRODUCTION

The amplitudes of miniature endplate potentials (MEPPs) recorded from the frog nerve muscle junction have been reported as being normally distributed (Fatt and Katz, 1952; del Castillo and Katz, 1954; Miledi 1960; Turkanis, 1973). Based on this finding of a single class of MEPPs, each MEPP is thought to represent the released contents of a single synaptic vesicle (del Castillo

and Katz, 1956). By using small muscle cells which give large MEPPs (Katz and Thesleff, 1957), we have found that 3% of the MEPPs form a second distinct mode whose mean amplitude is one-seventh that of the major mode (cf. Cooke and Quastel, 1973, on rat). Since low amplitude, low frequency MEPPs attributable to release of Schwann cell vesicles are seen after nerve section (Birks et al., 1960; Miledi and Stefani, 1970) we followed the effects of denervation on the MEPP amplitude distributions. Dennis and Miledi (1971) report small MEPPs from regenerating neuromuscular junctions which prompted us to examine developing junctions.

The mean MEPP amplitude at the frog neuromuscular junction appears to be stable over a spontaneous MEPP frequency range of several orders of magnitude (Katz, 1969) with various experimental conditions such as depolarization of the nerve terminal and changes in the chemical or osmotic environment (del Castillo and Katz, 1955; Katz, 1958, 1962). However, Mambri and Benoit (1964) have reported that MEPP amplitudes can be altered with a change in external calcium. The one procedure which has conclusively produced a marked decrease in MEPP amplitude has been prolonged repetitive nerve stimulation in the presence of hemicholinium (mammal, Elmquist and Quastel, 1965). The experience in our laboratory is consistent with the above observations when MEPP amplitude distributions are generated from muscle cells whose mean MEPP amplitude is 0.5 mV, which compares to the amplitudes of most reported studies. With the inherent increase in resolution using small muscle cell preparations (cf. Katz and Thesleff, 1957) we have found that an increase in spontaneous MEPP frequency by as little as five times produced as much as a 30% decrease in the mean MEPP amplitude. This work was presented to the Society of General Physiologists on 7 September 1973 (Gross and Kriebel, 1973).

#### METHODS

All experiments were performed on sartorius muscle cells from 1¼- to 2½-inch *Rana pipiens* and *Rana clamitans*. Tadpole experiments were performed on *Rana clamitans* in various stages of metamorphosis. Animals were doubly pithed and the sartorius removed by cutting along the fascia. The muscle was stretched to about normal length in a Plexiglas chamber with small hooks placed into the tendons. Hooks were placed into the fascia on each side of the muscle to separate the edge fibers so that the junctional regions could be visualized. The saline was composed of 106 mM NaCl, 2.1 mM KCl, 1.8 mM CaCl<sub>2</sub>, neostigmine bromide (10<sup>-6</sup> g/ml), and buffered to a pH of 7.4 with Trizma Base (2.0 g/liter) and Trizma HCl (5.3 g/liter) (Sigma Chemical Co., St. Louis, Mo.). The muscle chamber was mounted onto the stage of a compound microscope equipped with a × 20 refracting objective and a × 5 ocular containing a reticule for estimating muscle cell diameter. The bath temperature and rapid temperature changes were achieved by perfusing water of the desired temperature through a chamber located between the condenser and the preparation. Temperature changes of

20°C (from 13 to 33°C) were achieved within 15 s. Controls for most experiments were done at the temperature at which frogs were kept (13°C for winter and spring, 18°C for summer and fall). Nerve stimulation was accomplished by drawing the nerve into a suction electrode coupled to a stimulator through a stimulus isolation unit. Bath calcium was increased by adding an appropriate amount of isosmotic 20 × calcium saline (sodium was replaced) to achieve final concentrations of either 4 or 10 × normal calcium. The control ionic concentrations were reestablished by gently exchanging the bath contents five times with control saline over a period of 1 min.

Frogs to be denervated were anesthetized by placing them in tapwater containing 10<sup>-3</sup> g/ml MS 222 amphibian anesthetic (Sandoz, Inc., Hanover, N. J.). A 3–4-mm skin incision was made on the medial thigh exposing the sartorius nerve. Twelve mm of the proximal nerve was exposed and teased away from the neurovascular bundle. A 10-mm segment of the nerve was excised leaving a 1–2-mm distal stump. The proximal stump retracted between the adductor and triceps ensuring separation of the divided nerve ends. 0.1 cm<sup>3</sup> Neosporin ophthalmic ointment (Burroughs-Wellcome & Co., Tuckahoe, N. Y.) was injected under the skin through the incision. Skin incisions were healed within 3–6 days and denervated frogs were maintained up to 35 days with no sign of infection.

Conventional 3 M KCl micropipettes of 10–30-MΩ resistance were used. These were mounted on a sliding plate micromanipulator at an angle of about 30° to the floor of the bath and at a right angle to the muscle axis. The 10 × output of a high impedance DC amplifier was AC coupled to the vertical input of one beam (final gain, 1 mV/cm) of a dual beam oscilloscope usually set at a sweep speed of 100 ms/cm to monitor MEPPs. We also recorded representative MEPPs at greater sweep speeds (usually 10 ms/cm) in order to compare time-to-peak of small and large mode MEPPs. The output of the DC amplifier was also DC coupled to the external horizontal input (final gain, 10 mV/cm) of the second beam to monitor the muscle cell resting potential. The signals were recorded on moving film with an oscilloscope camera. MEPP amplitudes were determined by projecting records onto graph paper. The reading resolution was dictated by the noise (50–100 μV). By using cells 12–16 μm in diameter we were able to record up to 3 h with little or no change in resting potential though 10% changes were tolerated in some experiments. The histograms presented here were constructed from records of cells that had less than a 10% decrease in membrane potential. We found that a 10% change in resting potential did not mask the changes in the percentage of small and large mode MEPPs so we did not correct amplitudes (cf. Katz and Thesleff, 1957) for these small drifts in membrane potential. We have presented representative histograms based on 75 preparations each yielding from one to four edge fibers for study.

The total number of MEPPs generated with heat and calcium challenges which resulted in very high MEPP frequencies were estimated from fast oscilloscope sweep speeds. After strong heat challenges or repetitive nerve stimulation, we returned the preparation to control conditions which brought the spontaneous MEPP frequency back to control levels within 15 s. The amplitude distributions which show the effects of strong challenges were constructed from records after control frequency was reestablished. We were able to follow the changes in MEPP amplitude histograms during

the course of mild challenges on small muscle cells since the MEPP frequencies were still relatively low (cf. Kuno et al., 1971).

## RESULTS

### *Amplitude and Shape of Adult MEPPs*

Profiles of the MEPP amplitude distributions from fresh, low frequency, adult preparations revealed two striking and consistent features: (a) a population of small MEPPs with a mean one-fifth to one-seventh that of the major distribution and composing about 2–5% of the total population; and (b) a marked deviation of the major mode distribution profile from the expected Gaussian profile (Fig. 1 A) (cf. Liley, 1957). In medium cells (16–24- $\mu\text{m}$  diameter) and certainly in smaller cells (8–16  $\mu\text{m}$ ) the MEPPs contributing to the smallest mode formed a distinct distribution out of the noise; with the mean of the small mode ranging from 0.2–0.6 mV (large mode 1–4 mV, cf. Katz and Thesleff, 1957).

In low frequency cells the mean amplitude and amplitude distribution profile of the major mode would usually remain unchanged up to 3 h of recording. The configuration of the major mode profile was rarely symmetrical, usually showing shoulders; and many profiles showed a bi- or trimodal peak (Fig. 2 A). In those cells that showed an unexplained increase in frequency (possibly due to a change in stretch on the junction) of 5- to 10-fold, the mean of the major mode decreased during the generation of just a few thousand MEPPs. We also observed a few cases in which the mean increased (up to 30%) at the start of an experiment. These increases probably reflect recovery from high rates of transmitter release induced by the dissection and may be comparable to the recovery observed after a heat challenge or repetitive nerve stimulation discussed below.

Most of our experiments are from small edge fibers which do not show evidence of remote innervation. However, a few (10%) did show MEPPs with very slow rise times and of long duration indicating a remote junction (Fig. 3 A) (cf. Liley, 1957; Ginsborg, 1960). The records used here to construct the following amplitude histograms did not show evidence of a remote junction. We recorded MEPPs with fast oscilloscope sweeps and found that the time to peak of the small and large mode MEPPs was the same (Fig. 3 B). We also found some large MEPPs which had a longer time to peak but we ascribe this to the slightly asynchronous release of several quanta since we found a continuum of shapes ranging from obvious doublet MEPPs, to large MEPPs showing notches, to offsets on the rising phase, and finally very large MEPPs with normal time to peak (giants; cf. Liley, 1957). However, Negrete et al. (1972) ascribe long rise times to a process called spreading activation, i.e., large amounts of released acetylcholine saturate near receptors so that there is a delay as the excess acetylcholine diffuses to adjacent receptors.

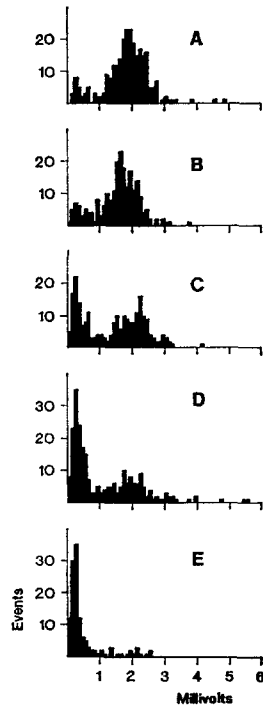


FIGURE 1

FIGURE 1. MEPP amplitude histograms showing effect of sequential heat challenges on subsequent amplitude distributions. For all histograms, MEPP frequency was about 1/s and preparation at 13°. (A) Control. Histograms B, C, D, and E, are each immediately after a 33° heat challenge. In B, note loss of 2.4-mV shoulder on profile A. Each challenge generated about 10,000 MEPPs. Resting potential: (A) -68, (B) -67, (C) -65, (D) -64, (E) -58 mV.

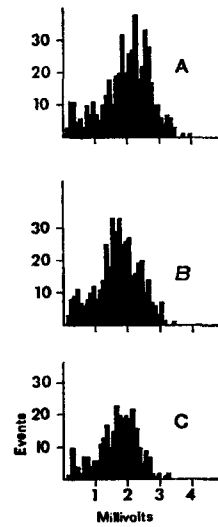


FIGURE 2

FIGURE 2. MEPP amplitude histograms showing effect of a moderate heat challenge on MEPP amplitudes. 14- $\mu$ m diameter cell. (A) Control, 14°; less than 1 MEPP/s, -84 mV. (B) First half of record during the 20° challenge, 10 MEPPs/s, -85 mV. Note loss of 2.7-mV peak on profile A. (C) Second half of record during the 20° challenge, 8 MEPPs/s, -90 mV. Note that the mean is under 2 mV, whereas the mean is well over 2 mV in A.

#### *Effect of Increased Temperature Challenge on Subsequent MEPP Amplitudes*

We found substantial changes in the MEPP amplitude histograms by increasing the bath temperature for 3–5 min which raised the MEPP frequency a 100-fold. We estimated the total number of MEPPs released during the challenge from representative fast oscilloscope sweeps. The number of generated MEPPs could only be determined to within a factor of two since the high rate of release produced many large MEPPs which probably resulted from the simultaneous occurrence of two or more. In 12- to 16- $\mu$ m diameter cells the temperature was adjusted to generate 10,000–30,000 MEPPs in a 3–5

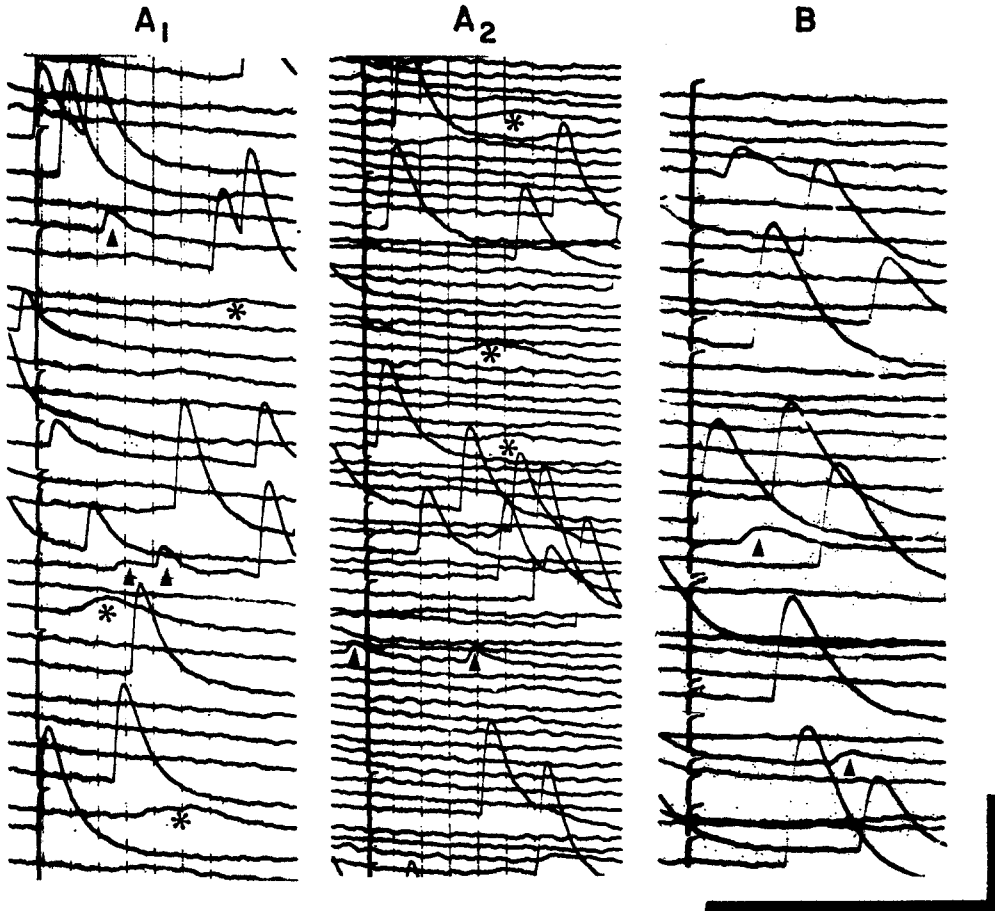


FIGURE 3. Sample records of MEPPs: (A) Doubly innervated muscle cell. Camera film speed is slower in  $A_2$  than in  $A_1$ . MEPPs generated by a remote junction have longer time characteristics and are indicated with an asterisk. Small mode MEPPs with shorter time characteristics are indicated with arrows. Mean of small mode MEPPs is one-fifth that of the major mode (3.0 mV). Small mode MEPPs composed 10% of amplitude histogram. Frequency was increased from less than 1/s to 5/s with  $4 \times$  calcium. Calibration: 200 ms and 4 mV;  $-92$ -mV resting potential.  $12\text{-}\mu\text{m}$  cell. (B) Rise times of small mode MEPPs (indicated with arrows) are similar to those of the larger MEPPs. Mean of small mode MEPPs is  $1/7$  that of the major mode (3.8 mV). Small mode MEPPs composed about 2% of the amplitude histogram. Calibration of high gain AC coupled trace is 100 ms and 4 mV. Resting potential is  $-90$  mV as measured from the right film margin,  $10\text{-}\mu\text{m}$  cell.

minute challenge. Since MEPP frequency is proportional to muscle cell diameter or junctional area (Kuno et al., 1971; Miledi and Potter, 1971) an equivalent challenge applied to a larger junction would necessarily generate a proportionally greater number of MEPPs. In many amplitude histograms

we noticed that the major mode was asymmetric due to shoulders (Fig. 1 A) or several peaks (Fig. 2 A). Amplitude histograms constructed after the initial challenge were notably missing the right-most shoulders and peaks seen on the major mode profile in the control plots. The mean of the major mode in histograms constructed after subsequent challenges did not shift farther to the left although the percentage of small mode MEPPs progressively increased until only the small mode remained (Fig. 1). The peak of the small mode after successive challenges appeared stationary even though the mean of the major mode initially shifted to the left.

In order to follow the initial configurational changes in the major mode profile we increased the MEPP frequency by one order to magnitude with moderate increases in bath temperature. By using small cells with very low control frequencies ( $< 1/s$ ) we were able to construct histograms from MEPPs recorded during the challenge. With a  $10 \times$  increase in MEPP frequency we found that the mean of the major mode shifted to the left (20–30%) within a few hundred MEPPs and then remained stationary. The reduced mean of the major mode in those amplitude profiles which were multimodal appeared to result from suppression of the right-most peak(s) or right shoulders (Fig. 2).

The amplitude profile would revert to the control profile within a few minutes even after a 100-fold increase in MEPP frequency providing that the time of the challenge was sufficiently short. This is shown in Fig. 4 in which 4,000 MEPPs were generated during a 1-min heat challenge and amplitude profiles were plotted for each successive time interval after return to control temperature (Fig. 4 B–I). This challenge was sufficient to transiently shift 30% of the MEPPs into the small mode (Fig. 4 B). Fig. 4 C–I show the progressive increase in proportion of large mode MEPPs after the frequency had returned to normal until the control profile (4 A) was reestablished by about 14 min.

#### *Effect of Calcium Challenge on Frequency and Subsequent MEPP Amplitudes*

We found that a  $4 \times$  calcium challenge usually doubled the frequency (Mambrini and Benoit, 1964) and a  $10 \times$  challenge increased the frequency 5–20 times. It is difficult to quantitate the effect across cells since calcium had a much greater effect on very low frequency junctions; e.g., when the control frequency was less than 0.5/s (small cells) a  $4 \times$  challenge sometimes increased the frequency 20-fold and a  $10 \times$  challenge sometimes increased the frequency 100-fold. As with a moderate increase in frequency produced with a heat challenge (Fig. 2) a frequency increase of 5–10  $\times$  with a moderate calcium challenge shifted the mean of the major mode to the left. In those control profiles which showed the major mode distribution to be multimodal, there appeared to be suppression of the far right peaks (Fig. 5). Prolonged

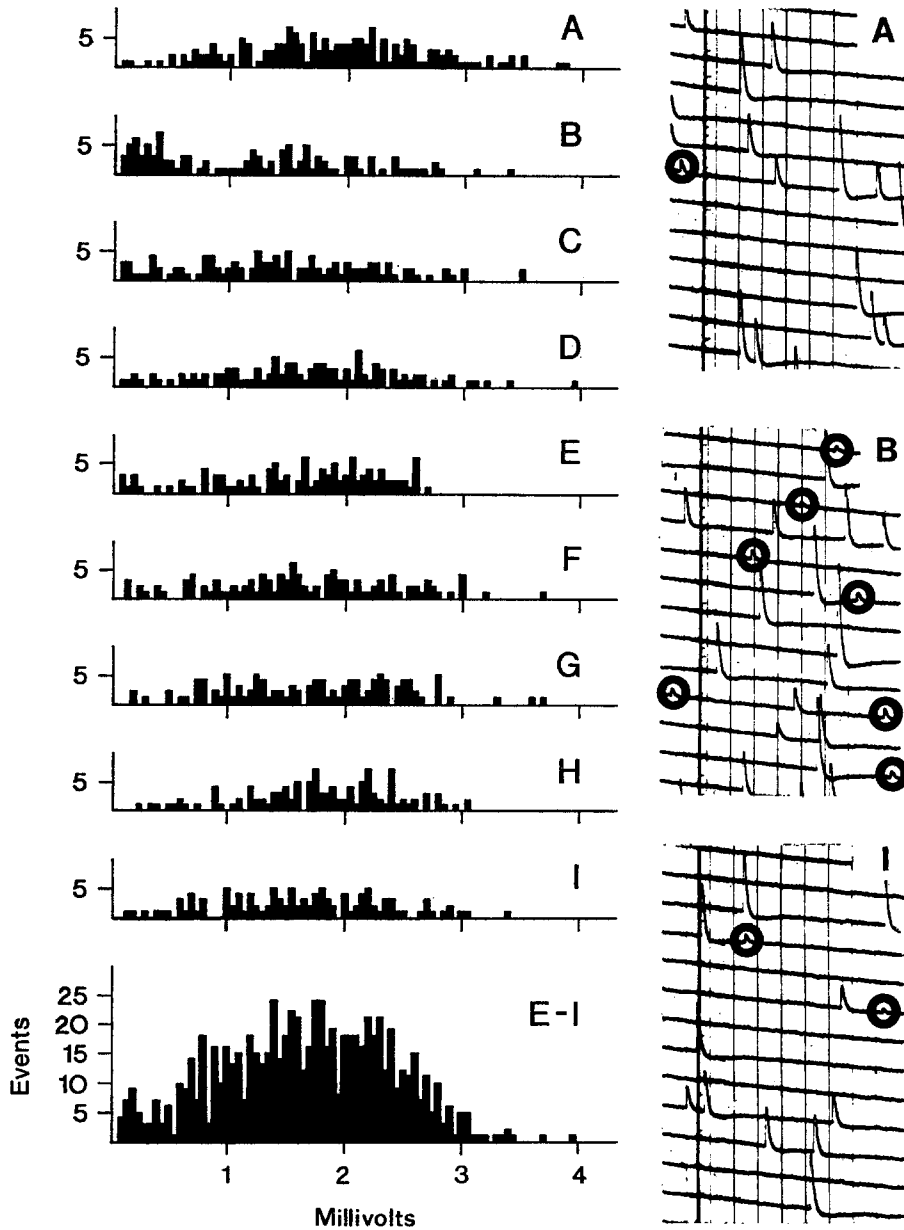


FIGURE 4. MEPP amplitude histograms showing effect of a 1-min temperature challenge ( $38^{\circ}$ ) which generated about 4,000 MEPPs on subsequent MEPP amplitudes (challenge between plots A and B). (A) Control  $18^{\circ}$ , about 1 MEPP/s, resting potential fell from  $-90$  to  $-85$  mV. (B-I) Sequential plots after the challenge. About  $18^{\circ}$  and about 1 MEPP/s. Resting potential  $-83$  mV in B,  $-85$  for other plots. Each plot contains 113 MEPPs which were generated in sequential periods (each period about 2 min). The last plot, E-I, shows small mode MEPPs which correspond to those in B just after the challenge. Small mode MEPPs are circled in the samples of records used to plot histograms. (1 s per sweep, 1.4 mV between traces,  $18^{\circ}\text{C}$ ). (Top) Control: sample of MEPPs used to plot histogram 4 A. (Middle) About 1 min after the heat challenge. Sample of MEPPs used to plot histogram 4 B. (Bottom) About 16 min after the challenge. Sample of MEPPs used to plot histogram 4 I.



moderate challenges resulted in an increase in the percentage of small mode MEPPs with usually no further shift in the major mode (Fig. 5 D).

The effect of a stronger calcium challenge was also similar to a heat challenge. This effect was most apparent in very low frequency cells which showed the greatest sensitivity so that a  $10 \times$  calcium challenge generated several thousand MEPPs within a 2-min period. Amplitude histograms plotted immediately after control frequency was reestablished ( $10 \times$  calcium saline was exchanged with normal saline) showed that one-third to one-half of the MEPPs were small mode MEPPs. 10–20 min after the challenge the amplitude distributions returned to normal. It is our impression that the effects of calcium and temperature are not strictly additive. Most of our calcium experiments were done at  $13^\circ$  which was the temperature at which we maintained the frogs. At this temperature most small cells had very low MEPP frequencies and a calcium challenge was very effective in increasing the frequency. We found that the increase in frequency with a room temperature challenge was usually sufficient to produce the left shift of the major mode so that a subsequent calcium challenge had little effect on the mean amplitude of the major mode and had a reduced and variable effect on MEPP frequency.

**DISCUSSION OF CALCIUM EFFECT** Mambrini and Benoit (1964) ruled out a possible postsynaptic effect of increased calcium since the response to electrophoretically applied acetylcholine remained unchanged. The observation that a temperature and calcium challenge are not strictly additive may be consistent with that found by Hubbard et al. (1971) on the rat. They found at least two groups of reactions involved in the spontaneous release of transmitter and the low temperature group was influenced by extracellular calcium whereas the high temperature group was not affected by extracellular calcium. With a  $4 \times$  calcium challenge Fatt and Katz (1952) also found a diminution of MEPP amplitude of 11% and Mambrini and Benoit (1964) report a 40% decrease in mean MEPP amplitude. Mambrini and Benoit also found that the amplitude increased to 120% with a reduction in calcium. Our work suggests that this calcium effect on MEPP amplitude is secondary to the change in MEPP frequency which influences MEPP amplitude (cf. Birks et al., 1968; Hubbard et al., 1968).

#### *Effect of Nerve Stimulation on Subsequent MEPP Amplitudes*

Nerve stimulation for periods of 1 min at 10 Hz caused a marked increase in the percentage of MEPPs contributing to the small mode (5% normal to 20–40% post stimulus) and a decrease in the mean amplitude (20–40%) of the major mode (Fig. 6). The first period of electrical stimulation also resulted in suppression of the far right shoulders (Fig. 6) or peaks as was found with increasing frequency via heat (Fig. 1) or calcium (Fig. 5) challenges. The major mode was not further shifted with subsequent stimulation although the percentage of small mode MEPPs increased. We also found a

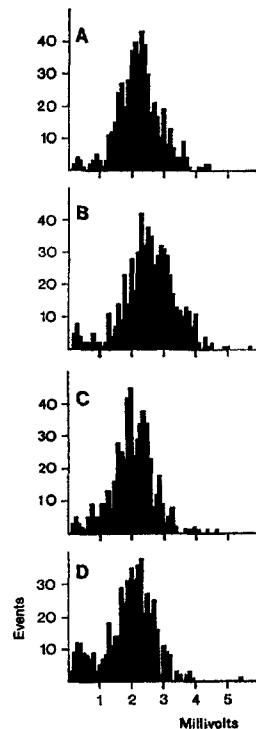


FIGURE 5

FIGURE 5. MEPP amplitude histograms showing the effect of a  $5 \times$  increase in frequency induced with a moderate calcium challenge ( $4 \times$  normal  $\text{Ca}^{++}$ ) on MEPP amplitude distributions.  $10\text{-}\mu\text{m}$  cell,  $13^\circ$ . (A) During first calcium challenge. 5 MEPPs/s. Resting potential  $-76$  mV. (B) Normal calcium less than 1 MEPP/s. Resting potential  $-77$  mV. Note the second peak at  $2.9$  mV. (C) First half of record during second challenge. 5 MEPP/s.  $-77$  mV. Note the loss of the  $2.9\text{-mV}$  peak. (D) Second half of record during second challenge. 5 MEPPs/s.  $-78$  mV. Note the increase in the percentage of small mode MEPPs.

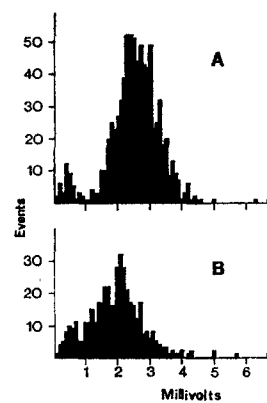


FIGURE 6

FIGURE 6. Effect of a 1-min period of nerve stimulation at 10 Hz on subsequent MEPP amplitudes.  $10\text{-}\mu\text{m}$  diameter cell,  $13^\circ$ . (A) Control. 1 MEPP/s: Resting potential fell from  $-75$  to  $-70$  mV during plot. (B) A few minutes after 1 min of 10 Hz nerve stimulation. Resting potential increased from  $-80$  to  $-84$  mV during plot. About 1 MEPP/s. Note that the major mode shows a 23% decrease in spite of the increase in resting membrane potential (cf. Katz and Thesleff, 1957).

few preparations which did not show a shift in the major mode with electrical stimulation but these did show an increase in the percentage of small mode MEPPs. The spontaneous MEPP frequency in these preparations was relatively high, leading us to suspect that the major mode had already shifted to the left. We found that histograms constructed after three to five 1-min periods of repetitive nerve stimulation (10 Hz) showed only small mode MEPPs.

*MEPP Amplitudes during Nerve Degeneration*

The effect of denervation on MEPP amplitudes was followed by sacrificing animals on sequential days after nerve section and comparing the denervated to the normal leg. MEPPs disappeared by the 10th–14th postoperative (PO) day, signaling the onset of the electrical quiescent period (Birks et al., 1960). The variability encountered in MEPP amplitude histograms across cells of any given preparation was great throughout the first PO week (cf. Birks et al., 1960). Most cells penetrated on the first PO day gave histograms similar to those found after a single moderate heat or calcium challenge, i.e., a bimodal distribution with the small mode comprising 10–15% of the total MEPPs and with the major mode mean about four to five times the small mode mean. Some cells, however, gave histograms in which the total MEPP population distributed evenly between the small and large mode. As degeneration progressed to the 6th PO day the situation progressively changed such that the common finding was a MEPP amplitude distribution in which the small mode comprised 50% or more of the total MEPPs and only a few distributions resembled normal profiles (Fig. 7). We also found a few amplitude profiles at around the 5th PO day that were rather flat and some were multimodal (Fig. 7 C) with the percentage of MEPPs in the various modes about the same. These “flat” distributions resemble those found in tadpole legs just before tail resorption (see next section).

Between the 5th and 8th PO day the MEPP frequency spontaneously increased 100-fold or more. In many cells this spontaneous increase developed during penetration and lasted an hour or so (cf. Birks et al., 1960). As this high frequency stage progressed, the mean MEPP amplitudes gradually fell until we were left with low amplitude, low frequency MEPPs. These remaining small MEPPs formed a symmetrical distribution out of the noise with a mean comparable to the mean of control cells of similar size (Fig. 7 E). The shapes of large and small mode MEPPs were the same as controls during the above stages of degeneration. Finally, even the small MEPPs disappeared such that no MEPP activity was seen during a 10-min period which is a criterion used to establish the period of electrical quiescence by Birks et al. (1960).

Up to about the 5th PO day these preparations responded to heat challenges with an increase in MEPP frequency and the proportion of small to large MEPPs increased with successive heat challenges just like normal preparations (Fig. 8). From the period of spontaneous increase in MEPP frequency to the “quiescent” period the MEPP frequency appeared relatively insensitive to heat and calcium challenges.

*MEPP Amplitude Distributions from Tadpole Leg Muscles*

MEPPs were recorded from tadpole leg muscles from early hindleg formation to complete tail resorption. It was difficult to maintain muscle cell

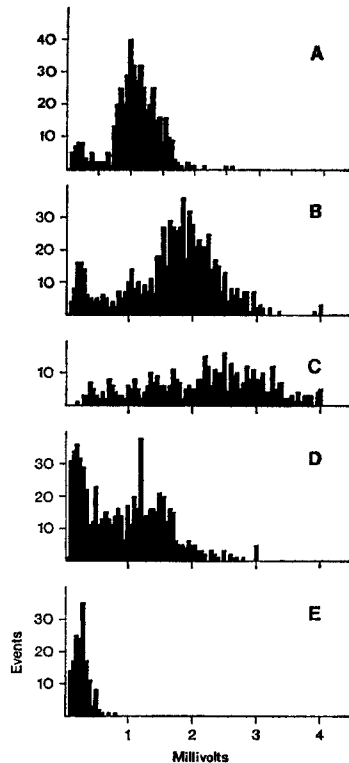


FIGURE 7

FIGURE 7. MEPP amplitude histograms of denervated preparations. (A-D) Spectrum of findings on the 5th PO day.  $13^{\circ}$ . (A)  $16 \mu\text{m}$ ,  $-78$  to  $-68$  mV during plot, about 2 MEPPs/s. (B)  $12 \mu\text{m}$ ,  $-90$  to  $-80$  mV. (C)  $12 \mu\text{m}$ ,  $-50$  mV. (D)  $16 \mu\text{m}$ ,  $-68$  to  $-61$  mV. (E) Histogram representative of the 8th to 11th PO day,  $15^{\circ}$ .  $12 \mu\text{m}$ ,  $-85$  to  $-87$  mV.

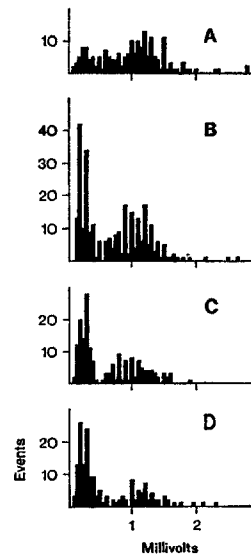


FIGURE 8

FIGURE 8. Effect of sequential heat challenges on a 4th PO day denervated preparation.  $12\text{-}\mu\text{m}$  diameter cell. All plots at  $13^{\circ}$  and with a MEPP frequency of about 1/s. The major mode is only three times the smaller mode indicating that a left shift has already occurred. Further support for this suggestion is that the major mode should be about 2.5 mV in a cell of this size. (A) Control. Resting potential  $-95$  mV. (B) Plot is after a 4-min,  $33^{\circ}$  heat challenge which gave a MEPP frequency of 40/s (generation of 10,000 MEPPs).  $-90$  mV. (C) Plot is after a second 10-min,  $33^{\circ}$  heat challenge which gave a MEPP frequency of 20/s (generation of 12,000 MEPPs).  $-95$  mV. (D) Plot is after a third, 2-min,  $33^{\circ}$  heat challenge which gave a MEPP frequency of 10/s. (generation of 1,200 MEPPs).  $-92$  mV.

resting potentials from very small legs but most MEPPs appeared to be small mode MEPPs. At later stages of hindleg development (before front leg emergence) the MEPPs formed multimodal amplitude distributions with the small mode dominating the histogram (9 A). The mean of the MEPPs composing the smallest mode corresponded to those recorded from adult muscle cells of similar size (about  $12 \mu\text{m}$ ). During tail resorption the per-

centage of larger MEPPs increased and profiles of amplitude distributions became flat or multimodal with each mode a multiple of the smallest (Fig. 9 B and C). Finally, after tail resorption, the adult histogram profile was obtained (Fig. 9 D).

At all stages the MEPPs had the same shape and rise times as MEPPs from adult preparations and were increased in frequency with a temperature or a calcium challenge although tadpole junctions were more sensitive to temperature or calcium challenges. As in adults, repeated challenges left

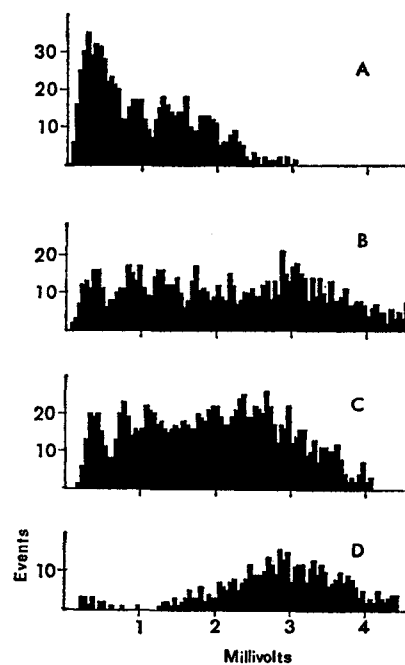


FIGURE 9. Representative histograms from various stages of metamorphosis. (A) Tadpole, just before front legs emerge.  $-75$  mV.  $12\text{-}\mu\text{m}$  diameter muscle cell. 1/s. (B) Beginning of tail resorption, 1/s. (C) Towards end of tail resorption, 1/s. (D) Tail completely resorbed. Small frog.  $-70$  mV, 1/s.

only small mode MEPPs. Fig. 9 shows the general course of the increase of the mean MEPP amplitude during metamorphosis even though we found variability within a given preparation. Larger percentages of small MEPPs may result from pithing which sometimes induced a few minutes of quivering and would thus be equivalent to a period of repetitive nerve stimulation.

#### DISCUSSION

Fatt and Katz (1952) demonstrated that MEPP amplitude histograms with mean MEPP amplitudes of  $0.5\text{--}1$  mV show a single normal distribution.

This observation strongly suggests a single class of MEPPs, each resulting from the release of a single packet or quantum of transmitter. Working with small cells which give larger MEPPs due to the greater input impedance, we found that MEPP amplitude histograms consistently show a second smaller mode whose mean amplitude is usually that of the major mode. We present five possible explanations which could account for the two sizes of MEPPs: double innervation, transmitter release from Schwann cells, diffuse release sites at a single junction, multiple classes of quanta, and a single quantal class which can be released singly or in multiples (cf. Dennis and Miledi, 1971).

*Postsynaptic Site of Currents Giving Small and Large MEPPs: Single Vs. Double Innervation*

Recording near one junction of a doubly innervated muscle cell (Katz and Kuffler, 1941; del Castillo and Katz, 1954; Liley, 1957) also gives a second class of small amplitude MEPPs. But the muscle cell cable properties which would account for the decreased amplitude also alter the time characteristics of remotely generated MEPPs. We recorded MEPPs at fast-sweep speeds during each experiment which show the rise time of our small and large mode MEPPs to be the same (Fig. 3 B). We did, however, find a few small muscle cells with two populations of small MEPPs, one population obviously generated by a remote junction as evidenced by the slow rise time and long duration of the MEPPs (Fig. 3 A). Del Castillo and Katz (1954) and Liley (1957) were also able to distinguish MEPPs generated by a remote junction without ambiguity. Another possible explanation for the two classes of MEPPs found in single innervated cells would be widely separated activity within a single long nerve filament (cf. Hubbard and Jones, 1973). But if the separation of activity were sufficient to give two classes of MEPP amplitudes it would be sufficient to enable differentiation of the two classes on the basis of time characteristics. Additional support for single sites is based on the fact that repeated high frequency MEPP generation shifts all the MEPPs into the small mode (Fig. 1). If remote junctions were the source of the small mode MEPPs, we would expect parallel changes in the two populations and not the observed shift from the large to the small mode. We feel that the above arguments localize the generation of fast, small, and large MEPPs to a single junctional region.

*Presynaptic Small Mode MEPP Generator: Nerve vs. Schwann Cell*

Birks et al. (1960) have recorded low frequency MEPPs of low amplitude and relatively long time constants at denervated junctions which emerge after a period of electrical quiescence. Electronmicrographs of denervated junctions show that Schwann cells containing vesicles have proliferated into

the regions previously occupied by nerve filaments which suggest that Schwann cells are the source of MEPPs at denervated junctions. In normal junctions, Schwann cell processes are regularly observed between the nerve filament and the muscle (Ceccarelli et al., 1972; Heuser and Reese, 1973) so it is conceivable that release of transmitter from Schwann cells could generate small mode MEPPs. However, Birks et al. (1960) have shown that the frequency of MEPPs recorded from denervated muscle is 1/100th that of MEPPs released from the nerve and that the frequency was not increased by a hypertonic or a temperature challenge. The frequency of MEPPs generated by Schwann cells was, however, increased with a hypotonic solution which had little effect on MEPPs released from nerve filaments. In normal and denervated preparations showing mainly small mode MEPPs after several heat challenges (Figs. 1 E, 8 D), MEPP frequency is still sensitive to temperature changes. Since the spontaneous shifts in amplitude histogram profiles seen during the early stages of degeneration (Fig. 8) are also similar to those experimentally induced in normal and denervated preparations by repeated challenges (Figs. 1, 8) we suggest that small mode MEPPs with rapid rise times in both normal and denervated preparations are generated by the nerve and not Schwann cell processes.

*Release Sites Giving Small MEPPs: Focal vs. Diffuse*

Ultrastructural studies of the frog nerve filaments reveal densely staining, perpendicular ridges spaced about 1  $\mu\text{m}$  apart located opposite the junctional folds (Birks et al., 1960; Ceccarelli et al., 1972, 1973; Heuser and Reese, 1973). Vesicles seem to funnel to these ridges which may represent the site of quantal release. Hubbard and Kwanbunbumpen (1968) have shown that membrane contacts between synaptic vesicles and axoplasmic membrane of the rat occur predominantly at the ridges which also suggests that this specialized cytoarchitecture represents release sites. Clark et al. (1972) have further evidence that the membrane near these ridges is the release site since application of black widow spider venom, which depletes the nerve of vesicles, causes separation of interridge surface from the postsynaptic element, whereas release ridges remain fixed opposite to the junctional folds and vesicles appear to fuse predominantly near the ridges. However, specialized release sites do not obviate the possibility that some quantal release occurs in the extraridge areas. Were this the case, one might expect some diminution of MEPP amplitudes secondary to dilution effects expected if the pulses of transmitter had to diffuse further to reach a receptor membrane; but we would expect this to give a continuum of MEPP amplitudes grading to the smallest size and not the discontinuum necessary to give a distinct small mode. We suggest that the site of release of transmitter resulting in small and large mode MEPPs is functionally the same.

*Origin of Small and Large Classes of MEPPs: Single vs. Multiple Sizes of Quanta*

We feel that these last two possibilities based on the vesicle hypothesis of transmitter packaging offer the best explanations for large and small mode MEPPs encountered in normal adult preparations: (a) There may be two sizes of vesicles or vesicles of similar size may be partially filled; and (b) vesicles of a similar size may be both singly and synchronously released.

**TWO SIZES OF VESICLES** Possibly the most obvious explanation would be two distinct sizes of quanta, (e.g., noradrenaline is released from large and small vesicles, Fillenz and Howe, 1970). Assuming a constant vesicular concentration of transmitter in the neuromuscular junction, the smaller vesicles would have a diameter half that of the larger vesicles to give a volume one-seventh that of the larger. This is not supported by vesicle size analysis which shows diameter distributions to be unimodal before and after 60% vesicle depletion (Heuser and Reese, 1973). We have shown that after 4–20 min of repetitive stimulation at 10 Hz (or a long heat challenge) almost all MEPPs are in the small mode at which time we see no obvious change in vesicle size or number (Rose et al., unpublished observations). If the bimodal MEPP distribution and the shift in population from the large to the small mode are to be explained on the basis of two sizes of vesicles one would expect that progressively increasing durations of electrical stimulation would show a progressively increasing proportion of small vesicles. Heuser and Reese (1973) found that after 1 min of nerve stimulation at 10 Hz the vesicle size appeared unchanged though the total number of vesicles was reduced by 25%; and, with 15 min of stimulation the vesicle diameter histogram remained unimodal, and contrary to what may be expected, the mean vesicle diameter increased by 10%. Ceccarelli et al. (1972) stimulated the nerve for 4 h at 2 Hz and found an 80% depletion of vesicles with no apparent change in vesicle size. This constancy of vesicle size is, however, in dispute since Korneliussen (1972) has found a decrease with stimulation. Perhaps the strongest physiological evidence against two sizes of vesicles is the multimodal plots from denervated preparations (Fig. 7 C) and tadpoles (Fig. 9 B and C) since six or seven vesicle sizes would have to be postulated.

**PARTIALLY FILLED VESICLES** Since the above arguments limit us to a single class of vesicle size, it seems logical to invoke partially filled vesicles to account for small mode MEPPs. Harris and Miledi (1971) also found a class of small MEPPs after treatment with botulinum toxin and suggest that these may result from either partial filling of vesicles or from retention of a fixed proportion of transmitter which is subsequently released. We propose that the first possibility would produce a continuum from filled to un-



filled vesicles which would skew the low end of the MEPP amplitude distribution and not form a distinct mode of small MEPPs (cf. Elmqvist and Quastel, 1965).

The second possibility depends on transmitter retention, recovery of vesicles, and subsequent release and warrants discussion in view of recent work on the fate of vesicular membranes. After incubation with horseradish peroxidase, Ceccarelli et al. (1972) found reaction products in vesicles which suggests that vesicles are recovered. Thus, a vesicle could release about five-sixths of its contents so that a subsequent release of the retained fraction would give rise to small MEPPs with an amplitude about one-seventh that of the major mode. However, this does not explain shifts in the mean of the major mode with the generation of only a few thousand MEPPs. In addition, Heuser and Reese (1973) propose that membrane is retrieved at a distance from the release sites as coated vesicles that coalesce into cisternae from which vesicles are subsequently formed. This method of membrane recycling would appear to release all of the vesicular contents in a single step. In addition, the multimodal histograms observed in denervated preparations (Fig. 7C) and in tadpoles (Fig. 9B and C) would be difficult to explain with a hypothesis based on the release of partially filled vesicles.

**UNITARY AND SYNCHRONOUS RELEASE OF A SINGLE VESICLE SIZE** The adult major mode profiles in most of our control plots markedly deviate from the expected Gaussian distribution (Figs. 2A, 5B, 6A) (cf. Liley, 1957; Martin and Pilar, 1964; Hubbard and Jones, 1973). Many of our normal large mode plots have a bi- or trimodal profile (Figs. 2A, 5B); and what superficially appears to be an "en bloc" left shift in the large mode with generation of a few thousand MEPPs (regardless of the kind of challenge) may be selective suppression of the right-most shoulders or peaks (Figs. 1, 2, 5). These shoulders and peaks suggest preferred multiples of quanta for synchronous release. Furthermore, the individual modes in the multimodal histograms recorded in denervated (Fig. 7C) and in tadpole muscle (Fig. 9B and C) appear to be integer multiples of the smallest mode. These observations suggest that MEPPs contributing to the larger modes may result from the synchronous release of quanta and that the smallest mode represents release of a single quantum.

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