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SUBUNIT COMPOSITION OF THE SPONTANEOUS MINIATURE END-PLATE CURRENTS AT THE MOUSE NEUROMUSCULAR JUNCTION

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SUMMARY

1. Adult, neonate and young mouse diaphragm muscle fibres were voltage clamped with a two-electrode clamp. Miniature end-plate currents (MEPCs) were recorded on magnetic tape and analysed with a computer. The MEPC amplitude, charge, rise time, time-to-peak, decay time constant and root mean square (r.m.s.) noise level were determined for each MEPC.

2. The MEPC amplitude and charge distributions showed integral peaks starting from zero. Peaks were enhanced by selecting MEPCs with uniform time characteristics, with low noise, with increased sample size, with a curve smoothing routine and/or with a selected bin size.

3. Integral peaks were found in histograms from neonate, young and old mice. The ratio of sub-MEPCs to bell MEPCs decreased during neonatal development.

4. The size of the peak intervals was the same in all preparations of the same developmental stage. The adult modal peak varied between 8 and 12 times the subunit value, but peak intervals were similar ($0{\cdot}44 \pm 0{\cdot}04$ nA).

5. Changes in the holding potential or the bath temperature, or addition of an anticholinesterase agent, changed the peak interval.

6. The number of peaks in the overall MEPC amplitude and area-to-peak (charge) histogram profiles were usually the same.

7. Integral peaks on MEPC amplitude profiles, notches and steps on the MEPC rising phase and changes in the overall MEPC profiles are explained by a subunit composition of the quantum of transmitter release.

INTRODUCTION

The initial studies concerned with the amplitude distributions of spontaneous miniature end-plate potentials (MEPPs) show a normal distribution indicating a uniform class of events (Fatt & Katz, 1952; del Castillo & Katz, 1954; Boyd & Martin, 1956; Liley, 1956). Dennis & Miledi (1971, 1974) described a second, smaller class of MEPPs which skew into the background noise in the frog reinnervated junction, and Harris & Miledi (1971) reported similar small MEPPs with botulinum

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toxin treatment (termed skew MEPPs). Small MEPPs (sub-MEPPs) were also found in the normal rat diaphragm muscle (Cooke & Quastel, 1973) and normal frog muscle (Gross & Kriebel, 1973). With substantially increased signal-to-noise ratios, Kriebel & Gross (1974) and Kriebel, Llados & Matteson (1976) demonstrated that the skew MEPP distributions form a mode and that some MEPP amplitude histograms show integral peaks. Kriebel, Llados & Matteson (1982) found that integral peaks on the evoked quantal potential amplitude distributions matched those on the spontaneous MEPP amplitude distributions. The skew MEPP class is not evoked in reinnervated preparations (Dennis & Miledi, 1971) and normal adult preparations (Kriebel *et al.* 1976; Bevan, 1976; Kriebel, 1978). Wernig & Stirner (1977) found integral peaks and Wernig & Motelica-Heino (1978) demonstrated that the peaks on frog MEPP amplitude distributions became either larger with an anticholinesterase agent or sharper by selecting focal MEPPs. The anticholinesterase experiment was confirmed on the mouse diaphragm by Carlson & Kriebel (1985). Integral peaks form the basis of the subunit hypothesis of the quantum of transmitter release (Kriebel & Gross, 1974; Kriebel *et al.* 1976, 1982; Wernig & Motelica-Heino, 1978; Kriebel, 1978). The integral peaks of MEPP amplitude distributions are remarkably uniform and do not appear to increase in variance as predicted from the observed sub-MEPP variance (see Bevan, 1976; Katz, 1977, 1978). Matteson, Kriebel & Llados (1979, 1981) modelled theoretical distributions of MEPPs composed of subunits with a variance of the observed sub-MEPP distribution; and, as predicted by Katz (1977, 1978) they found that the variance smoothed the amplitude profile after the first five or six peaks. However, when the contribution of the background noise and measurement errors were subtracted from the sub-MEPP variance, eight to twelve integral peaks were found on the MEPP amplitude distribution (Matteson *et al.* 1981).

The purpose of this study was to voltage clamp the mouse neuromuscular end-plate with a two-electrode clamp to measure miniature end-plate current (MEPC) amplitudes and time characteristics with interactive computer programs. Under adequate spatial clamp conditions the MEPCs are independent of the passive electrical properties of the muscle fibres so it is possible to compare MEPCs across fibres and preparations. With standard conditions MEPC amplitude distributions showed the same peak intervals and these intervals changed with treatments (change of temperature or holding potential, or added anticholinesterase agent) that altered the postsynaptic channel characteristics. These observations support the 'subunit hypothesis' of the quantum of transmitter.

METHODS

Hemidiaphragms (seventy-five) of 1- to 10-day-old neonate, young and adult mice (most preparations) were used. The voltage clamp techniques were standard (Erxleben & Kriebel, 1988). Standard conditions were with a holding potential of -140 mV, a bath temperature of 30 °C to promote tissue stability, and a 25 mM-Ca^{2+} saline to enhance electrode sealing. The excess Ca^{2+} reduced the mean MEPC by about 25% but promoted electrode sealing and increased MEPC frequencies. The MEPCs were recorded on magnetic tape and were subsequently digitized so that MEPC amplitudes, rise time, time-to-peak, decay phase and root mean square (r.m.s.) baseline noise level were determined for each signal (Erxleben & Kriebel, 1988). Amplitudes were measured to 1024 classes and classes were combined to yield 60–120 bins in the histograms. Signal-to-noise ratios were between 50:1 and 100:1.

RESULTS

General comments

Seventy-five junctions of the 100 hemidiaphragm preparations studied had adequate signal-to-noise ratios, at least 10^3 MEPCs for amplitude distribution studies, and a constant mean MEPC amplitude for detailed analyses. Because of various difficulties in achieving adequate data which yield amplitude histograms showing integral peaks, and the fact that some investigators have not found integral peaks while specifically looking for them, general comments concerning our voltage-clamping procedure are relevant (Carlson & Kriebel, 1985). In some of the first experiments with a normal Ca^{2+} concentration saline it was necessary to record for up to 1 h in order to collect enough MEPCs and we found in some preparations that the mean MEPC progressively increased. This change in quantal size turned out to be an artifact attributed to an increase in tonicity with evaporation. A change in quantal size of a few per cent is enough to mask integral peaks (see Kriebel *et al.* 1982). We avoided this problem with a high-Ca^{2+} saline which increased the MEPC frequency from less than 1/s (30 °C) to 10/s. To achieve adequate signal-to-noise ratios the bath was not perfused. Maximal sample sizes of $2–4 \times 10^3$ isolated MEPCs were all that could be practically recorded with a 50:1 or better signal-to-noise ratio which was required to show the sub-MEPC mode and produce MEPC amplitude histograms with peaks. We chose isolated MEPCs for this study so that the MEPC rising and falling phases could be analysed without extrapolation procedures.

Amplitude distribution of neonate, young and adult MEPCs

We found that the amplitude distributions of 1-day-old neonate mice had many sub-MEPCs (see Muniak, Kriebel & Carlson, 1982) and that the percentage of bell MEPCs increased with maturation until the sub-MEPCs composed only a few per cent (Fig. 1*A*). The MEPC amplitude histograms from 1- to 3-week-old mice show that the skew MEPC class represented 10–20% (Fig. 1*B*). The first peak of the skew MEPC class (or the skew MEPC mode) represents the sub-MEPCs (Fig. 1*A*; Matteson *et al.* 1981; Kriebel *et al* 1982). Most of the studied junctions are from adult preparations because it was much easier to maintain stable recordings in order to collect adequate sample sizes. During maturation, the overall variance of the MEPC amplitude profile decreases, which is another reason for using adult mice (Fig. 1*C*). We found that $2–4 \times 10^3$ MEPCs were needed to show three to five central, integral peaks when the MEPC amplitude histogram was broad (Fig. 2*A*). On the other hand, 10^3 MEPCs were adequate to show two or three peaks in the central region of the adult distribution when the overall profile was narrow, because of the relatively large sample sizes per bin in the central region of the distribution (Fig. 2*B* and *C*). We have found that forty events per bin yields adequate sample sizes to show central peaks, provided that there are at least forty bins to the modal MEPC bin (the sample size in Fig. 2*C* is marginal). Since the percentage of sub-MEPCs was usually low (1%) in adult preparations, most histograms do not show a clear sub-MEPC mode (sub-MEPC mode in Figs 1*A* and 1*B* and 2*C*; the skew class is represented in Figs 1*A–C* and 2*C*; see Carlson & Kriebel, 1985, for sub-MEPPs in adult preparations).

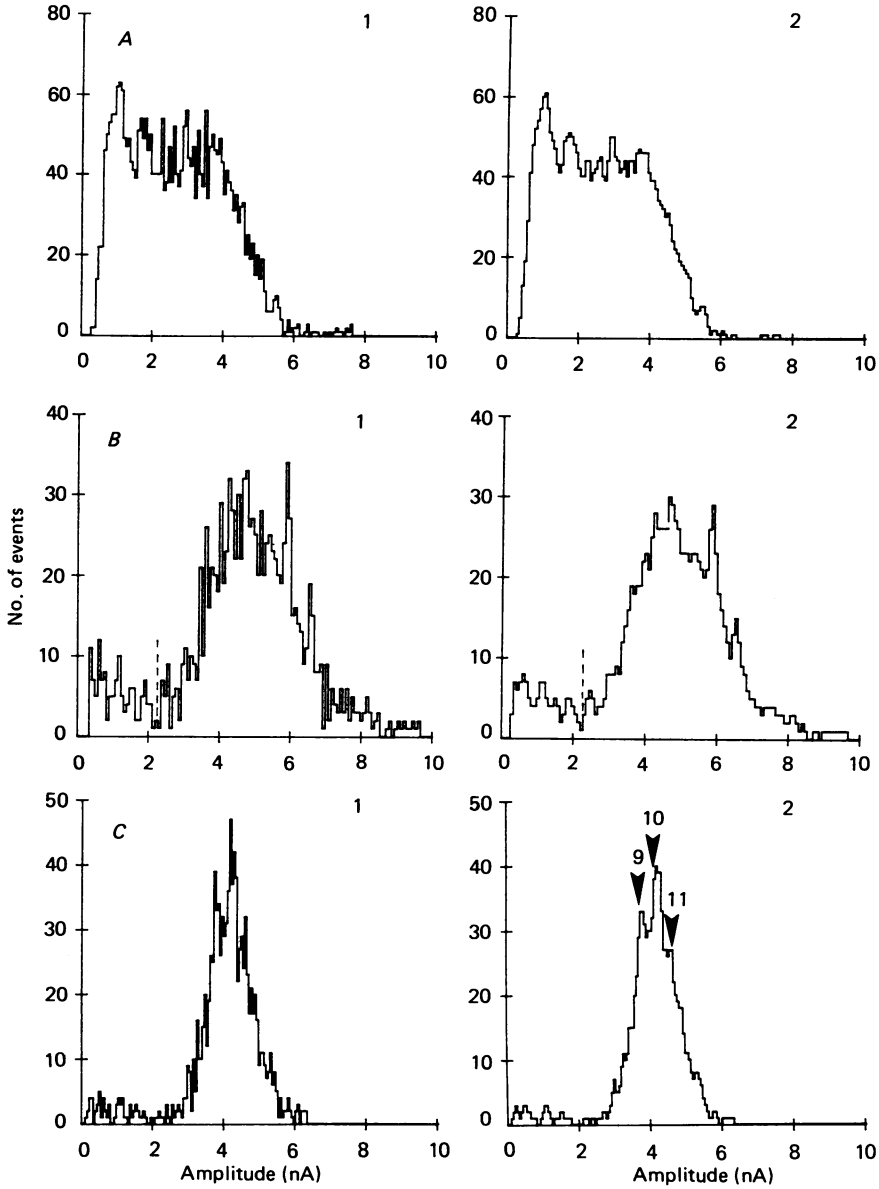

Fig. 1. Sample MEPC amplitude histograms from mice of different ages (*A–C*), showing raw (1) and filtered (smoothed) data (2). The smoothing routine was a three-bin moving average. Note that the smoothing routine diminishes the single-bin 'fingers' that result from random variations of small sample size. *A*, neonate mouse. 1, raw data. Holding potential (HP) −100 mV; 30 °C. The discontinuity between skew and bell MEPCs is not clear. $2{\cdot}9 \times 10^3$ MEPCs. 2, smoothed data. *B*, 3-week-old mouse. 1, raw data. Note discontinuity between skew and bell MEPCs (dashed line). HP −140 V; 30 °C; physostigmine. $1{\cdot}2 \times 10^3$ MEPCs. 2, smoothed data. Sub-MEPCs are first peak of the skew class. *C*, young adult. 1, raw data. Note small percentage of skew MEPCs. HP −140 mV; 30 °C. $1{\cdot}4 \times 10^3$ MEPCs. 2, smoothed data. Note three central peaks that are integral multiples starting from zero.

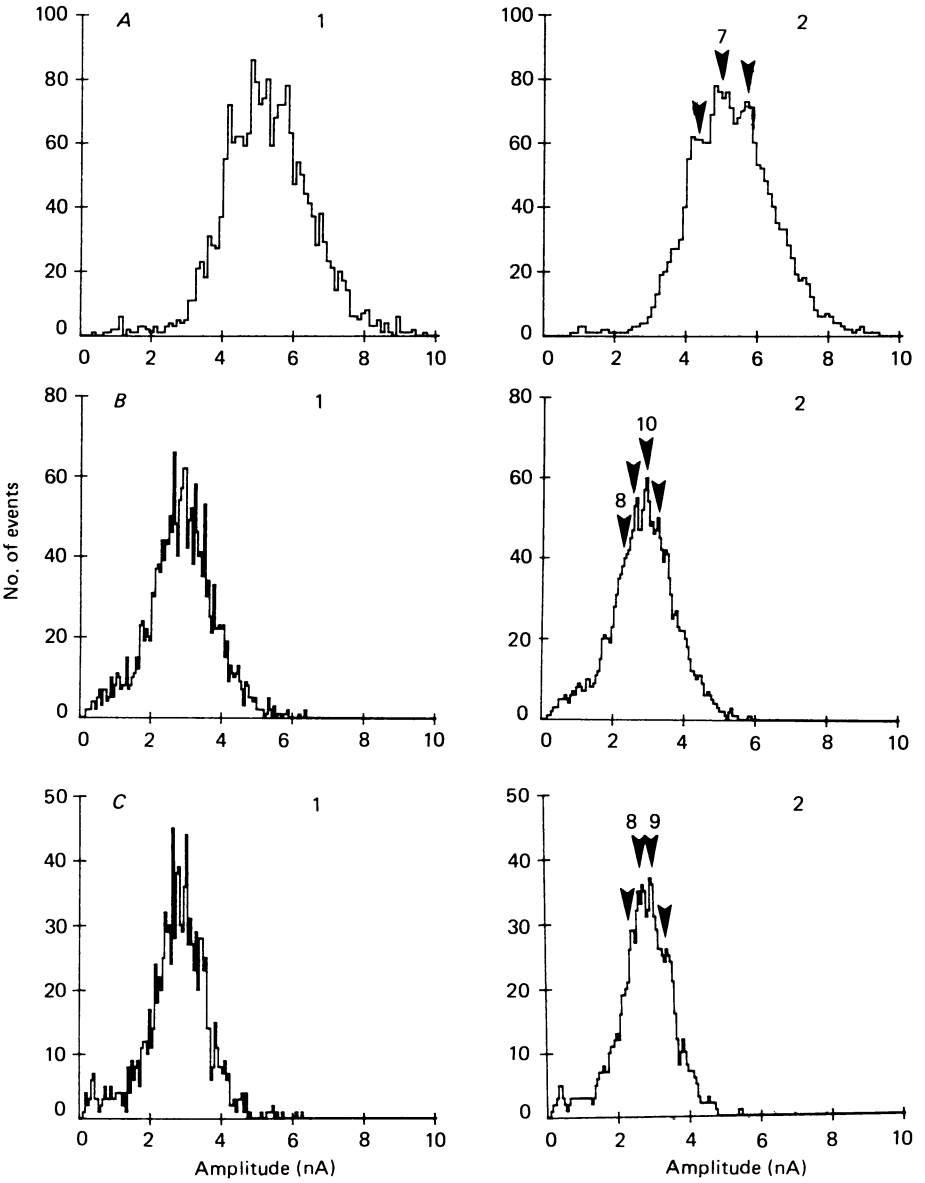

Fig. 2. Typical MEPC amplitude histograms from three adult mice (*A*, *B* and *C*), showing raw (1) and smoothed (2) data. These were selected to show range of distributions with integral peaks and suggestive peaks. Arrow-heads are at integral multiples starting from zero. Numbers indicate multiples of subunit. *A*: 1, raw data. Not enough small MEPCs to show skew class. Peaks are apparent. 30 °C, HP −140 mV; physostigmine. $2{\cdot}0 \times 10^3$ MEPCs. 2, smoothed data. *B*: 1, raw data, note the ragged appearance of the profile due to relatively small numbers of events in some of the bins. $2{\cdot}0 \times 10^3$ MEPCs. 2, integral peaks emerge with smoothing. *C*: 1, raw data: sample size relatively small so random variations in events per bin are dominant. $1{\cdot}1 \times 10^3$ MEPCs. 2, smoothed: the presence of central peaks is questionable.

Spontaneous change in MEPC quantal size

Even with carefully controlled recording conditions such as temperature, low background noise and low, stable holding current we sometimes found that the mean MEPC changed during the recording period (Kriebel *et al.* 1976). These changes were usually noted immediately after dissection and during long recording periods, but MEPC amplitude distributions show the same integral peaks for the entire period of recording (Fig. 3). Addition of the first and second halves of the data show the same peak intervals (Fig. 3*C*), indicating that the average peak number changed during the course of the experiment.

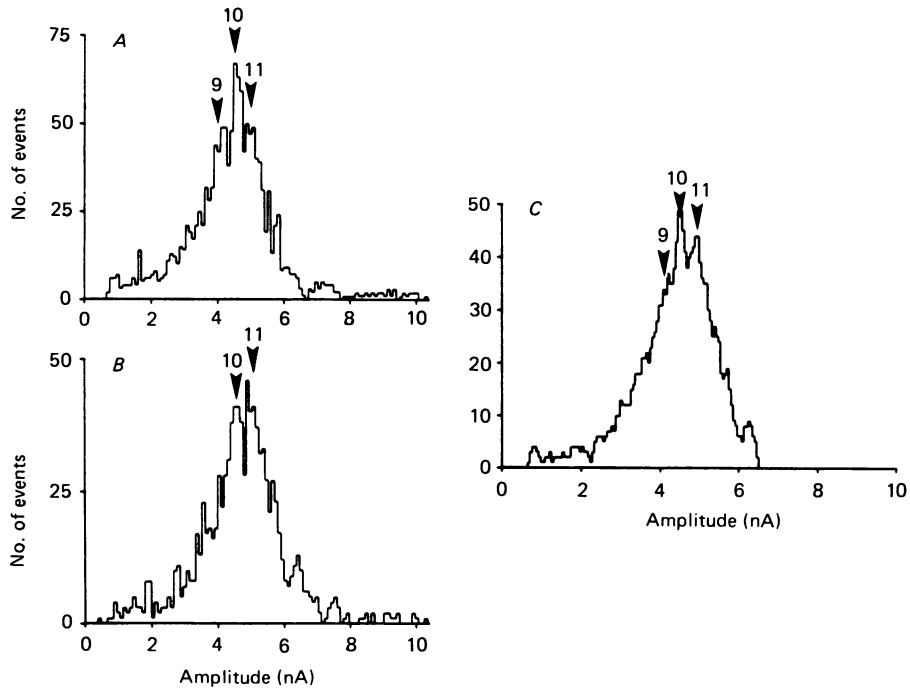

Fig. 3. The MEPC amplitude histograms of the first and second halves of the recording period. −140 mV HP, 30 °C. Raw data: change in mean MEPC but note value of peak intervals remains constant. The peaks are indicated with arrows and the number is the number of subunits. *A*, first $1{\cdot}4 \times 10^3$ MEPCs. Note three central integral peaks. *B*, second 10^3 MEPCs. Note two prominent central peaks that have the same integral value starting from zero as in *A*. *C*, total. $2{\cdot}4 \times 10^3$ MEPCs. Smaller bin size than in *A* and *B*. The peak integral values are the same in all three histograms. (Note change in abscissa scale which was necessary to show the smaller bin size.)

Influence of bin size and curve smoothing on profile of the MEPC amplitude distribution

The choice of bin size is important because small bins result in too few events per bin which yield a ragged MEPC amplitude profile (Figs 1*A*1 and 1*B*1) due to the influence of random variations in sample size, whereas too large a bin containing many events may yield a histogram without adequate resolving power to show clear integral peaks (Fig. 5). At least four bins between peaks are necessary to resolve the

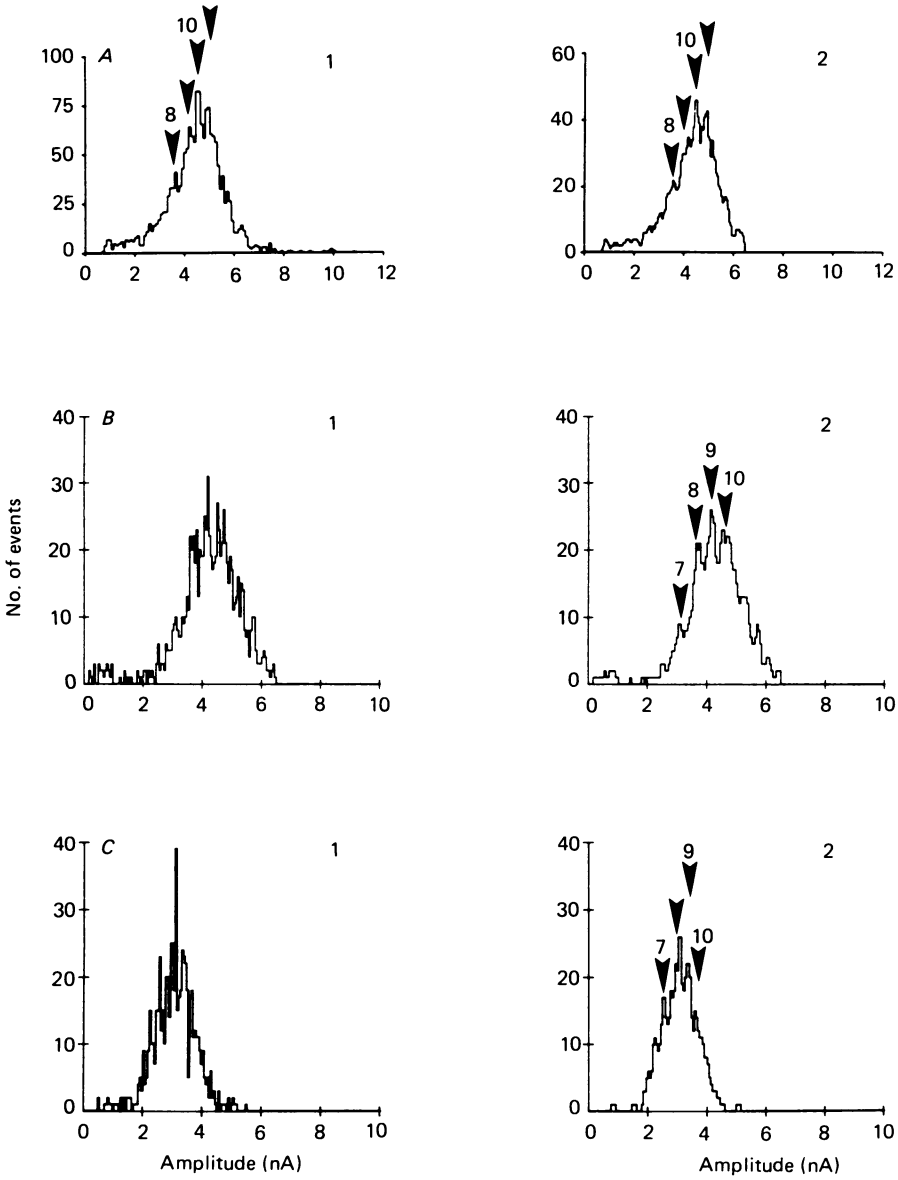

Fig. 4. Influence of bin size and/or the curve-smoothing routine on MEPC amplitude profiles. Arrow-heads are spaced at integral multiples starting from zero. Numbers indicate multiples of subunit. *A*: 1, smoothed; larger bin size. Note that each peak is composed of about 3·5 bins and that the peaks are in the same position as in 2. 2, raw data; smaller bin size. Note that each peak is composed of about five bins and that overall shape and position of peaks is same as in 1. $1{\cdot}5 \times 10^3$ MEPCs. *B*: 1, raw data; relatively small sample size. $9{\cdot}5 \times 10^2$ MEPCs. 2, smoothed data. Three integral central peaks emerge. Note skew class. *C*: 1, raw data; small sample size. $6{\cdot}5 \times 10^2$ MEPCs. Ragged appearance due to 'fingers' composed of single bins. 2, smoothed data. Note integral peaks which start from zero which are not apparent in 1.

peaks. Visual determination of peak intervals was found to be as reliable as maximum likelihood estimates (Matteson *et al.* 1981) so peaks were fitted by eye so that intervals started from zero. The bin size was chosen to be at least two times the r.m.s. value of the background noise level. It would be meaningless to resolve amplitudes to a value less than the r.m.s. background noise level (Wong & Redman, 1980). We did find that the sample size could be reduced and the peaks retained by selecting only MEPC amplitudes with signal-to-noise ratios of 120:1. Usually, our sample sizes were large enough so that central integral peaks were apparent with the appropriate bin size. The MEPC amplitudes were measured to 1024 classes and we found that combining classes by four to seven yielded amplitude distributions with central peaks. The integral peaks remained in the same position with different bin sizes and/or curve smoothing routine (Figs 4 and 5). A three-bin moving-average curve-smoothing (filtering) routine helped to pinpoint the peak values and to determine the integral value between the peaks which started from zero (Fig. 4*A*, *B* and *C*). Too few MEPCs per bin yield ragged MEPC profiles (Fig. 5*A*1) but integral peaks emerge with filtering (Fig. 5*A*2); also, an increase in the bin size shows peaks in raw and filtered data (Fig. 4*B* and *C*), whereas large bins smooth the integral peaks of the raw data (Fig. 5*D*1) and these peaks disappear with filtering (Fig. 5*C*2 and *D*2).

The MEPC charge and amplitude distributions

We also plotted net MEPC charge (total area of MEPC) and MEPC area-to-peak histograms and found that these distributions show the same number of integral peaks as the MEPC amplitude histograms and the same overall profile (Fig. 6).

Effect of an anticholinesterase agent on MEPC amplitude profiles

An anticholinesterase agent made the MEPCs larger and greatly prolonged their falling phase (Erxleben & Kriebel, 1988). However, the number of integral peaks remained the same and only the peak interval increased (Fig. 7*A*; Carlson & Kriebel, 1985). With the concentrations we used there was probably no change in single-channel conductance and only an anticholinesterase action (Fiekers, 1985).

Effect of an increase in voltage clamp holding potential on MEPC amplitude profiles

Increasing the holding potential of the muscle fibre from −140 to −170 mV increased the MEPC amplitudes and the number of bins between central, integral peaks but did not alter the shape of the MEPC amplitude profile or the number of central peaks (Fig. 7*B*1 and *C*1 at −140 mV, Fig. 7*B*2 and *C*2 at −170 mV). We found this experiment difficult to perform since the fibres tended to be unstable at −170 mV.

Effect of temperature on amplitude profiles

Elevating the bath temperature to 38 °C (mouse temperature) proved to be a difficult experiment because of spontaneous contractions of the preparation. However, we did succeed in performing three experiments at 38 °C and −140 mV and two experiments at 38 °C and −170 mV and obtained larger peak intervals (Fig. 8)

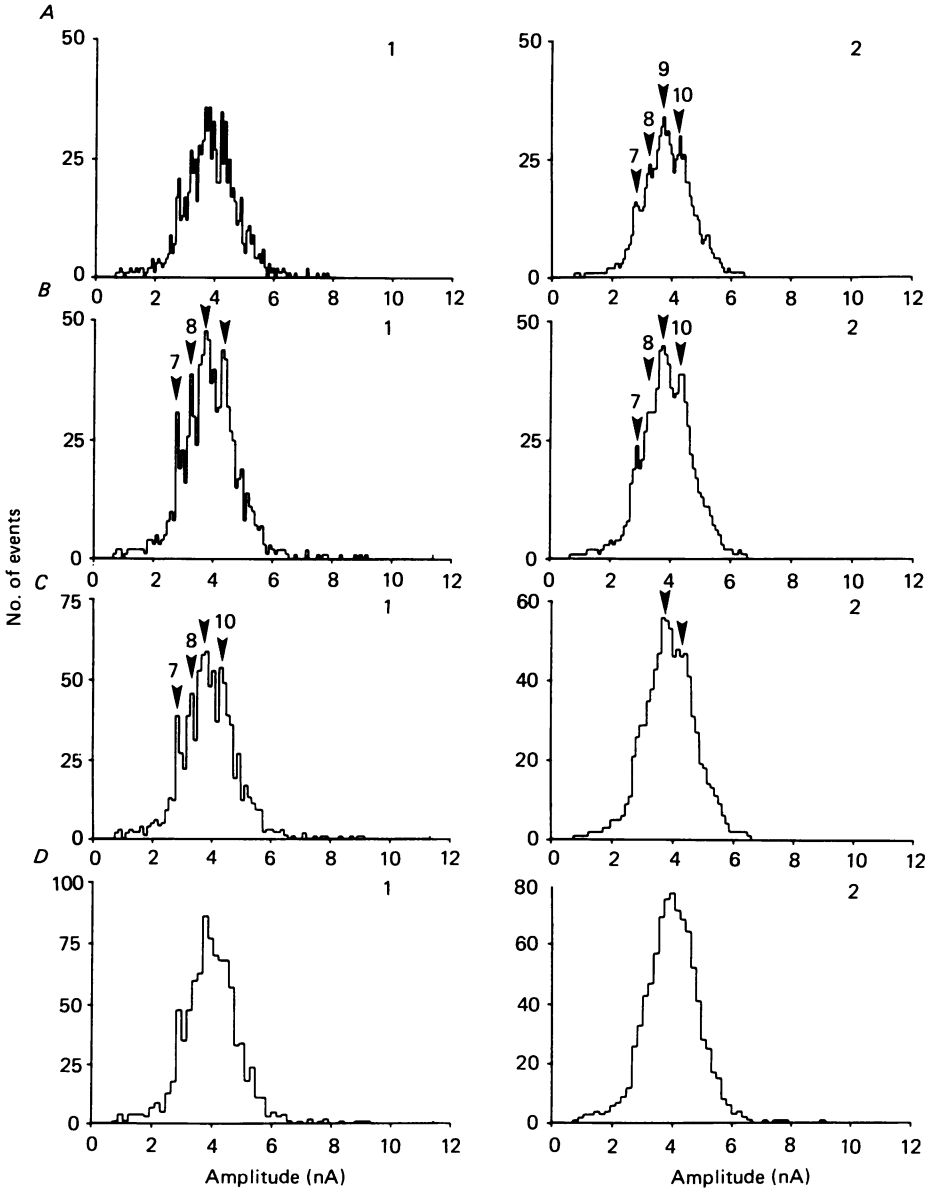

Fig. 5. Influence of both curve smoothing and bin size on MEPC amplitude histogram profiles. The MEPC amplitudes were measured to 1024 classes. The classes were then combined in groups of 5, 7, 9 or 13 to form the histograms. $9{\cdot}1 \times 10^2$ MEPCs. *A*: 1, raw data; classes combined in groups of 5 for each bin. Ragged appearance of profile. Relatively small sample size for each bin. 2, smoothed data. Note integral peaks which start from zero. *B*: 1, raw data: classes combined in groups of 7 for each bin. Note presence of integral peaks with same spacing as in *A*2. 2, smoothed data. Same integral peaks are present and number 8 is represented as a shoulder. *C*: 1, raw data: classes combined in groups of 9 for each bin. Peaks show same integral value as in *A* and *B*. Sample size for central peaks now adequate. 2, smoothed data. Side peaks disappear because there are not enough bins between peaks. *D*: 1, raw data: classes combined in groups of 13. Peaks not in evidence. Bin size is too large to reveal peaks. 2, smoothed data.

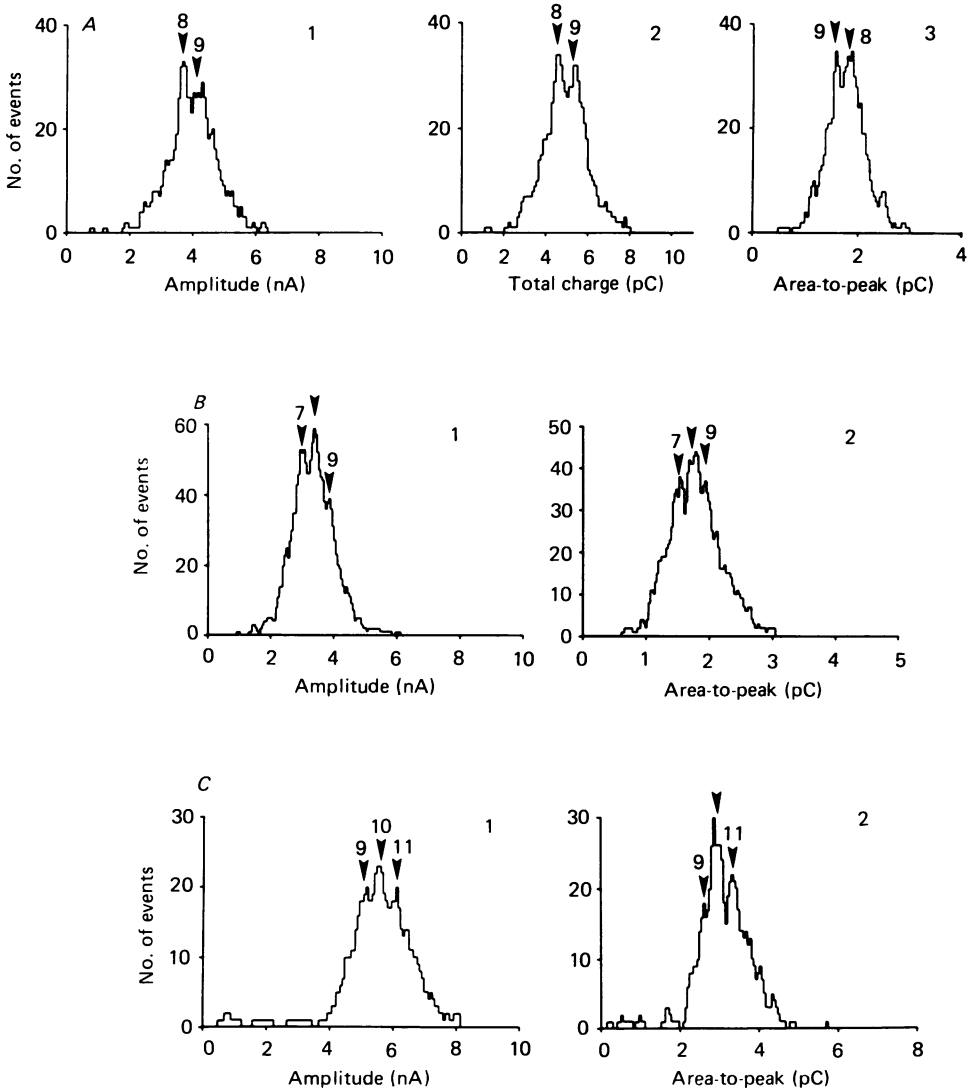

Fig. 6. Comparisons of MEPC amplitude histogram to area-to-peak current histogram and total current histogram. Three junctions. *A*: 1, MEPC amplitude histogram; smoothed data. $9{\cdot}2 \times 10^2$ MEPCs. 2, MEPC total charge histogram, smoothed data (same integral peaks as in *A*1). 3, MEPC area-to-peak histogram (same integral peaks as in *A*1). *B*: 1, MEPC amplitude histogram, smoothed data. $1{\cdot}9 \times 10^3$ MEPCs. 2, MEPC area-to-peak histogram, smoothed data. *C*: 1, amplitude, smoothed data. $7{\cdot}3 \times 10^2$ MEPCs. 2, MEPC area-to-peak, smoothed data. Profiles of *B*2 and *C*2 are slightly skewed to the right.

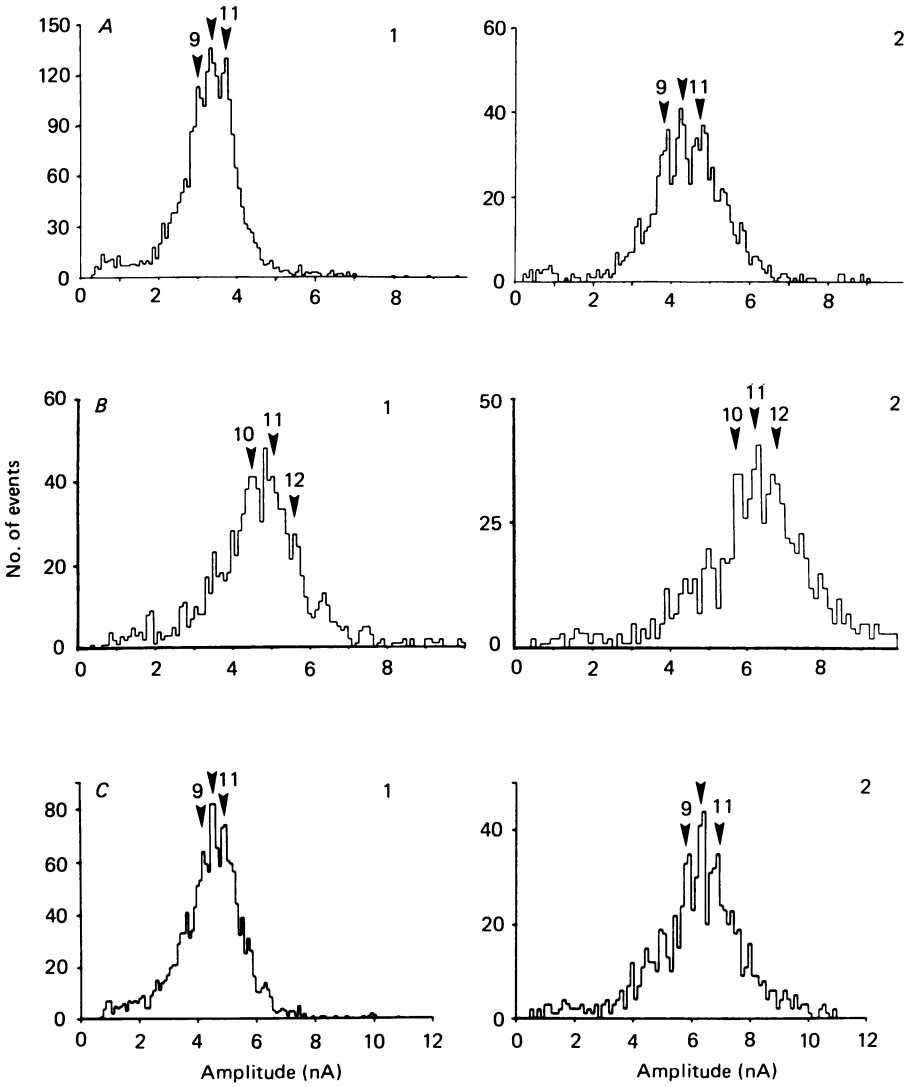

Fig. 7. Effect of physostigmine and different holding potentials on MEPC amplitude histogram profiles. Arrow-heads are at integral values starting from zero. Raw data. *A*: 1, control, 20 °C, holding potential −140 mV. Note large number of events in central portion of the histogram and three central peaks. $2{\cdot}5 \times 10^3$ MEPPs. 2, after physostigmine. The three central peaks represent the same peaks as in 1 but with a larger peak interval. $1{\cdot}2 \times 10^3$ MEPPs. *B*: 1, holding potential −140 mV. 10^3 MEPCs. 2, holding potential −170 mV. Note that profiles of both *B*1 and *B*2 are similar with modal peak at number 11. $1{\cdot}2 \times 10^3$ MEPCs. *C*: 1, holding potential −140 mV. $2{\cdot}4 \times 10^3$ MEPCs. 2, holding potential −170 mV. $1{\cdot}1 \times 10^3$ MEPCs. Note that the profile of 1 and 2 are the same with the same three central peaks (9, 10 and 11).

than under control conditions. Cooling the bath from our control condition of 30 °C to either 25 or 20 °C resulted in amplitude histograms with smaller peak intervals (Fig. 8).

Size of peak intervals

We were able to compare the control peak intervals with those obtained after changing the postsynaptic characteristics in fourteen preparations. Control peak intervals of the fourteen preparations (−140 mV holding potential, 30 °C high-Ca^{2+}

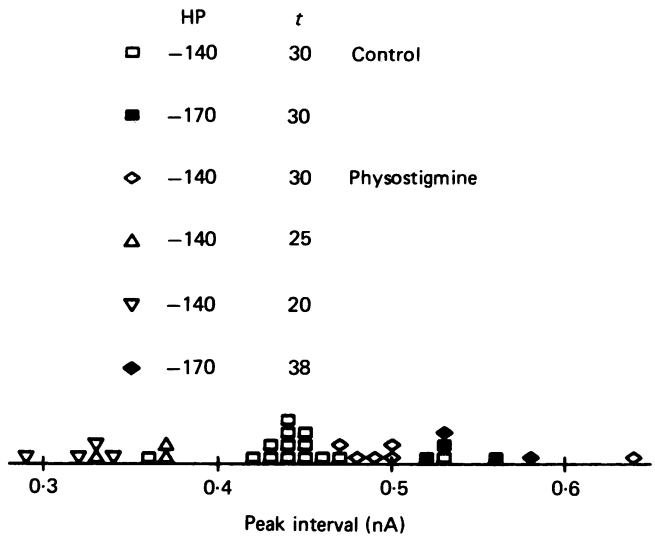

Fig. 8. Size of integral peak intervals from fourteen control junctions and eighteen experimental conditions which were selected for clearly defined peaks at integral multiples starting from zero. Peak intervals given ±1 S.D. The peak intervals measured under experimental conditions are significantly ($P < 0{\cdot}01$) different from the control values. HP, holding potential. *t*, temperature. □, control values (0·44 ± 0·04 nA) are from fourteen preparations maintained at 30 °C, holding potential −140 mV and 25 mM-Ca^{2+} saline. ◇, six preparations were treated with physostigmine (0·51 ± 0·06 nA). ■, three junctions were voltage clamped to −170 mV (0·54 ± 0·02 nA). ◆, two junctions were voltage clamped to −170 mV along with an increase in bath temperature to 38 °C (0·56 ± 0·04 nA). △, the temperature was dropped to 25 °C in three preparations (0·36 ± 0·02 nA). ▽, the temperature was dropped to 20 °C in four preparations (0·32 ± 0·02 nA).

saline) were 0·44 ± 0·04 nA. The peak intervals were changed in all fourteen preparations with experimental conditions which were known to have a postsynaptic effect on either the single-channel characteristics or driving force (Fig. 8). Cooling the bath decreased the peak interval, whereas increasing the holding potential, increasing the bath temperature, or adding an anticholinesterase agent increased the peak intervals (Fig. 8). All experimental conditions produced peak intervals that were significantly different from the controls and changed the intervals as predicted by the change in postsynaptic characteristics.

Steps and notches on the rising phase of the MEPCs

Some preparations generated MEPCs with steps, notches or breaks on the rising phase and there were too many to represent chance coincidence (Fig. 9; Kriebel & Stolper, 1975; Kriebel, Llados & Carlson, 1980). There were many MEPCs with a step on the rising phase the size of a sub-MEPC, and the size of the signal from the step

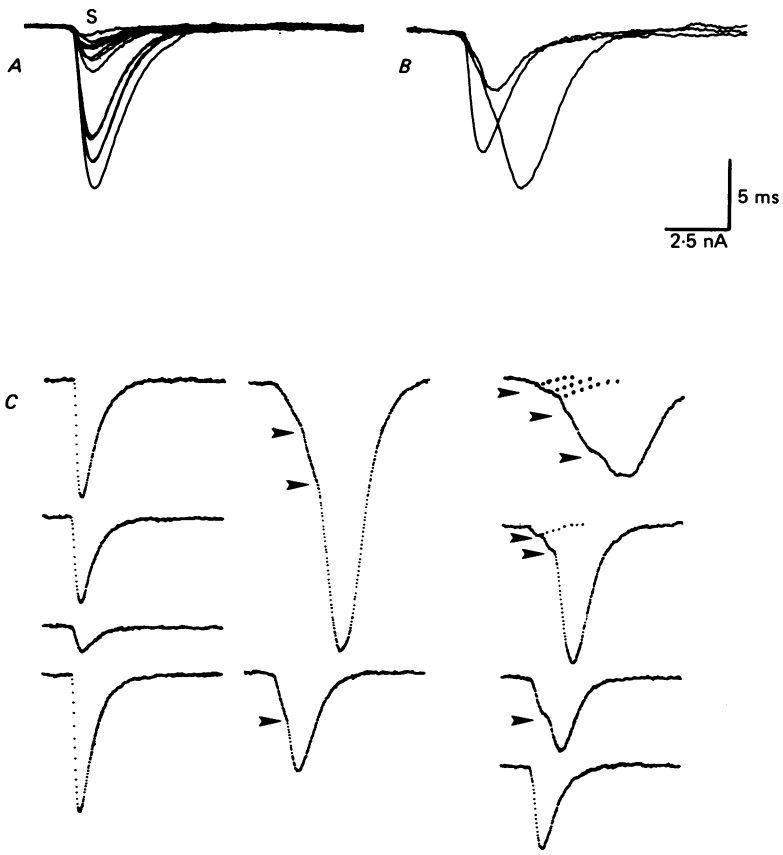

Fig. 9. Sample MEPCs. *A*, superimposed MEPCs showing two classes. The smaller cluster of MEPCs comprise the skew MEPCs of which the smallest is a sub-MEPC (see Fig. 1*B*2). *B*, three superimposed MEPCs showing two atypical MEPCs with slow rising phases but normal falling phase (termed 'tents'). *C*, series of MEPCs, some with normal, smooth rising phases. Those rising phases with steps, notches or breaks are indicated with arrows. The dotted lines show the size of sub-MEPCs.

to the peak was less than that of the average bell MEPC (Fig. 10). Two MEPCs occurring slightly out of phase due to chance would yield a signal with steps or breaks on the rising phase, but these chance occurrences would generate amplitude histograms of the steps or notches with the same distribution as those of the single MEPC.

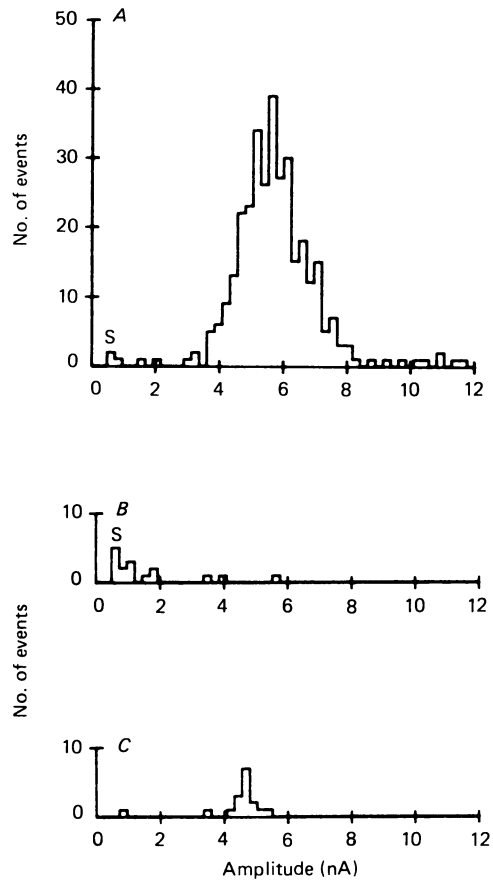

Fig. 10. MEPC amplitude histogram and amplitude of breaks on the rising phase. HP −160 mV. *A*, MEPC distribution. Sub-MEPCs are at S. *B*, amplitude of break on rising phase. Note that the amplitude of most breaks is of the sub-MEPC size. *C*, amplitude of signal after first break. Note that the mean is smaller than the average MEPC by about 3 bins (i.e. the size of the sub-MEPC).

DISCUSSION

Effect of postsynaptic change on peak intervals in MEPC amplitude distributions

The MEPP amplitude histograms published by Kriebel & Gross (1974) and Wernig & Stirner (1977) for a frog sartorius preparation and Kriebel *et al.* (1978, 1982) for a mouse diaphragm preparation show integral peaks starting from zero in the central part of the distributions. Integral peaks are also demonstrated with banding patterns on multiexposed photographs of MEPPs which were used to trigger the oscilloscope trace (Kriebel, 1978; Vautrin & Mambrini, 1981). Moreover, the integral peaks do not change with successive samples (Kriebel *et al.* 1976, 1982; Carlson & Kriebel, 1985) so they do not arise from chance variations in small sample sizes (cf. Magleby & Miller, 1981). Wernig & Motelica-Heino (1978) and Carlson & Kriebel (1985) found that an anticholinesterase agent increased the peak interval but not the number of

peaks. The purpose of this study was to show that a number of central peaks persisted after a change in postsynaptic characteristics. Peaks on the sides of the distributions are not clearly defined because random variations due to small sample size are large in the side bins (Magleby & Miller, 1981). We verify that the integral peaks remain in the same position and are accentuated with a selected bin size for a given set of data, and we demonstrate that the peaks are clarified with a curve-smoothing routine (filtering). Some distributions containing 10^3 MEPCs exhibited three sharp peaks in the central region providing that the overall distribution was narrow. We found that a sample size of forty events per bin is minimal for showing integral peaks and that there must be over forty bins to the mode (see Carlson & Kriebel, 1985; cf. Bevan, 1976). We used adult preparations which generate few skew MEPPs and this explains why the skew class is not well represented and why there are no clear peaks on the skew distribution (see Kriebel *et al.* 1976, 1982; Matteson *et al.* 1981).

The accuracy of the subunit model has been evalutated from many selected histograms by a χ^2 goodness-of-fit test, and the generalized likelihood ratio test was used to test the subunit model against a reduced bimodal model (Matteson *et al.* 1981). The fit produced by the subunit model was accurate for 90% of the sixty-eight histograms tested and the subunit model was significantly better than the bimodal model (skew and bell MEPPs) for 93% of the sixty-eight histograms tested (Matteson *et al.* 1981). Of the seventy-five junctions examined using the voltage clamp technique reported here, over half produced amplitude distributions with central integral peaks starting from zero, equivalent to those modelled by Matteson *et al.* (1981). The uniform MEPC amplitude peak interval of fourteen junctions which was 0·44 nA with a standard deviation of only 0·04 nA strongly argues against chance variations in samples, because chance groupings from different preparations would yield MEPC amplitude histograms with different peak intervals (Magleby & Miller, 1981). Moreover, the experimental treatments which altered the postsynaptic membrane characteristics significantly changed the peak intervals as predicted. With the sample sizes we were able to achieve in this study, we know that random variations can generate peaks in about 5% of the cases (F. Llados & M. E. Kriebel, unpublished data). Thus, to obtain by chance the eighteen experimental histograms from the fourteen preparations used for controls would be essentially impossible.

Subunit hypothesis

Many of the distributions presented here show sharp peaks that do not appear to broaden with increasing peak number. However, the MEPC amplitude histograms are very similar to published MEPP amplitude histograms, and these fit a subunit model based on a subunit variance and background noise (Matteson *et al.* 1981; Kriebel *et al.* 1982). We feel that the studies of Wernig & Motelica-Heino (1978), Vautrin & Mambrini (1981), Carlson & Kriebel (1985) and those presented here strongly support the observation that amplitude distributions of spontaneous quanta show integral peaks. Csicsaky, Papadopoulos & Wiegand (1985) and Vautrin (1986) have analysed their amplitude histograms with peaks, as well as those published by Kriebel and co-workers with Fourier analysis (Kriebel *et al.* 1976, 1982), and report substantial evidence for preferred amplitudes (but see Magleby & Miller, 1981). The observation that the MEPC (not MEPP) peak interval is constant

throughout junctions (provided experimental conditions are identical) supports the presynaptic origin of the subunit. A postsynaptic origin seems very unlikely for several reasons (but see Vautrin & Mambrini, 1981). It has been found that MEPCs have uniform time characteristics regardless of their amplitude (Erxleben & Kriebel, 1988). Thus, a lower receptor density or a different (extrajunctional) receptor can be excluded as an explanation. Secondly, there is a discontinuity in the amplitude profile between bell and skew classes, although both classes have the same peak intervals (Carlson & Kriebel, 1985). Thirdly, the unitary evoked potential is of the bell class and the histogram shows the same peak intervals as on the MEPP distribution (Kriebel *et al.* 1982). Fourthly, the number of peaks and their size can be changed with treatments that alter rates of release (elevated Ca^{2+} ions, Ca^{2+} ionophore, temperature change, nerve stimulation, La^{3+} ions, botulinum toxin and β-bungarotoxin (Kriebel & Gross, 1974; Kriebel *et al.* 1976; Kriebel, 1978; Kriebel *et al.* 1980; Llados, Matteson & Kriebel, 1980; Kriebel & Florey, 1983)). During the initial stages of various treatments, the quantal size may not change. Finally, it is important to stress that changes in mean MEPP amplitude usually occur after the release of large numbers of quanta and after the initial high rates of release and thus cannot be attributed to changes in postsynaptic membrane. On the other hand, botulinum toxin treatment blocks the bell MEPPs after the release of only a few hundred quanta, leaving the sub-MEPPs (Kriebel *et al.* 1976).

The MEPCs which are atypically shaped and those with a foot or break on the rising phase can be explained by the subunit hypothesis. The mechanism that normally synchronizes the release of the subunits may deteriorate and spread the subunits in time. Histograms of the amplitudes of breaks or steps on the rising phase of MEPPs reflect the skew MEPP distribution and not the overall MEPP amplitude distribution as would be expected from random nearly coincident events (Fig. 10; Carlson, Kriebel & Muniak, 1982).

Morphological correlate of the subunit of the physiological quantum

Kriebel & Gross (1974) initially proposed that the sub-MEPP may reflect the release of a synaptic vesicle. Wernig & Stirner (1977) proposed that the 'active zone' could function as a unit in such a way that the synchronous release of several vesicles would generate the quantum; this idea was supported with the observation that the number of release sites found with a focal electrode matches the number of 'release ridges' and not the number of touching vesicles.

The observation that there are two classes of MEPPs (bell and skew classes) presents a problem because there is only one class of synaptic vesicle based on diameter (Kriebel & Florey, 1983; Kriebel, Hanna & Muniak, 1986). Moreover, in preparations that generate mainly sub-MEPPs either following nerve stimulation (Rose, Pappas & Kriebel, 1978) or with La^{3+} (Kriebel & Florey, 1983) the remaining vesicles are of control size. During synapse formation there is only one vesicle class, regardless of the ratio of skew to bell MEPPs. Thus, there is one vesicle class based on diameter but the mechanism of evoked release normally utilizes quanta of the bell class (Bevan, 1976; Kriebel, 1978; Kriebel *et al.* 1982) and botulinum toxin selectively blocks the bell MEPP quanta first (Kriebel *et al.* 1976). Morphological evidence supports the univesicular hypothesis of the physiological quantum. Heuser,

Reese, Dennis, Jan, Jan & Evans (1979) found some correlation between the number of quanta released with 4-aminopyridine and the combined numbers of fused synaptic vesicles and particle clusters. Katz & Miledi (1979) also potentiated evoked release and calculated that the number of released quanta was greater than the number of vesicles in contact with the presynaptic membrane. There is evidence against bound vesicular acetylcholine for the basis of subunits because Fuldner & Stadler (1982) found that *Torpedo* vesicular acetylcholine is free in the synaptosome. The overall variance of the bell MEPP class of quanta is 30% (Fatt & Katz, 1952) but that of the subunit (and sub-MEPP) is only 10–18% (Wernig & Motelica-Heino, 1978; Matteson *et al.* 1981). This small variance of the subunit size places a constraint on the molecular and/or organelle presynaptic basis of the subunit.

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