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TEMPERATURE EFFECTS ON SPONTANEOUS AND EVOKED QUANTAL SIZE AT THE FROG NEUROMUSCULAR JUNCTION[1]

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Abstract

The amplitudes, time integrals, and half-decay times of miniature endplate currents (MEPCs) and of endplate currents (EPCs) were measured in preparations in low Ca^{2+}-high Mg^{2+} Ringer, in which the probability of the evoked release of more than one quantum was low. The measurements were made at 5 to 7, 11, and 15°C. There is no consistent difference in these properties of evoked and spontaneously released quanta at any of the temperatures. I. S. Cohen and W. Van der Kloot ((1983) J. Physiol. (Lond.) *336:* 335–344) compared the effects of temperature changes on MEPCs and on the endplate currents evoked by set amounts of iontophoretically applied ACh. The results suggested that the quantity of ACh/quantum was relatively unaffected by changes in temperature. This conclusion can now be extended to quanta released by nerve stimulation.

One possible mechanism for quantal release is the diffusion of transmitter from the cytoplasm of the presynaptic terminal outward by way of a gated channel (del Castillo and Katz, 1954a; Isräel and Dunant, 1975; Marchbanks, 1975). Three observations suggest that the gated channel is unlikely to be the mechanism for spontaneous quantal release. (*1*) Miniature endplate potential (MEPP) or miniature endplate current (MEPC) amplitudes are not appreciably altered when the membrane of the nerve terminal is depolarized by electrotonus (del Castillo and Katz, 1954b; Cooke and Quastel, 1973) or by elevated extracellular K^+ (Linder and Quastel, 1978; Cohen and Van der Kloot, 1983). The change in membrane potential would affect the flux of acetylcholine (ACh) if it passed through a channel as a cation. (*2*) MEPP amplitudes are not appreciably affected by massive alterations of the osmotic pressure of the extracellular solution (Van der Kloot, 1978). The shrinking or swelling of the nerve terminal should at least momentarily alter the [ACh] in the terminal and, therefore, also change the efflux through a gated channel. (*3*) The quantity of ACh per quantum is probably the same over a temperature range from 4° to 25°C (Cohen and Van der Kloot, 1983). The open time of known gated channels is markedly temperature dependent.

These observations make it unlikely that spontaneous quantal release occurs by way of a gated channel and favor the vesicle hypothesis, which is consistent with each of the three results. However, in some instances there are notable differences between quanta released spontaneously and those released following stimulation. For example, there are differences in the amplitudes of MEPPs and uniquantal endplate potentials (EPPs) at regenerating neuromuscular junctions (Dennis and Miledi, 1974) and at central synapses in the hatchet fish (Bennett et al., 1976). False transmitter appears to be incorporated more rapidly into quanta that are released following stimulation than into those released spontaneously (Large and Rang, 1978a, b). Possibly evoked and spontaneous quantal release employ different mechanisms (Tauc, 1982). To test this possibility we have compared the effects of temperature on evoked and spontaneous release at the frog neuromuscular junction; recall that our previous results with MEPPs and ACh iontophoresis showed that the quantity of ACh per quantum appears to be independent of temperature. We have extended these results to stimulated release by comparing the size of MEPCs and uniquantal endplate currents (EPCs) at different temperatures. To avoid the potential difficulty of comparing MEPCs with multiquantal EPCs, we worked in high Mg^{2+}-low Ca^{2+} solution, so that almost all of the evoked releases contained a single quantum.

Materials and Methods

The experiments were performed on the sciatic nerve-sartorius muscle preparation of the frog, *Rana pipiens*. The bathing solution contained (in millimolar concentration): 120 NaCl, 2 KCl, 0.20 to 0.27 $CaCl_2$, 10 $MgCl_2$, and 4 *N*-Tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) at pH 7.4. The preparation was pinned in a dish with a base of silicone rubber. The dish was on a temperature-controlled Peltier plate. The temperature of the solution near the muscle was monitored with another thermistor. The temperature did not vary by more than ±0.1°C. The nerve was fitted into a stimulating electrode that was then filled with paraffin oil, to keep the nerve moist. The nerve was stimulated with square pulses of 0.8-msec duration at 0.5 to 1.0 Hz.

EPCs were recorded by the two-electrode voltage clamp method using a Dagan 8500 voltage clamp. The microelectrodes were first beveled to DC resistances of 4 to 6 megohms (Lederer et al., 1979). The holding potentials were −100 or −140 mV. Figure 1 shows the method employed for digitizing the EPCs and MEPCs and for recording them

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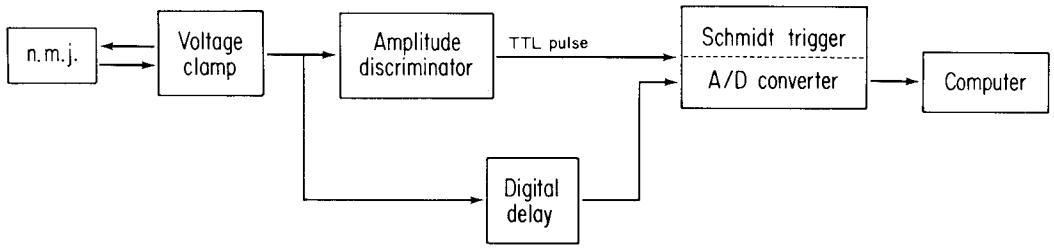

Figure 1. A diagram of the recording setup. A WP amplitude discriminator was used to detect the appearance of a MEPC. The pulse from the discriminator was used to start the A/D converter. The EPC entering the A/D converter was delayed for 40 msec by a Dagan digital delay. Therefore, a section of the signal preceding the upstroke of the MEPC was recorded. When recording EPCs a pulse from the stimulator triggered the Schmidt trigger on the A/D converter. *N. M. J.*, neuromuscular junction; *TTL*, transistor-transistor logic.

on magnetic discs. The sampling was at either 125 or 250 μsec/point. Usually 200 EPCs were recorded, followed by 100 MEPCs and then an additional 100 to 200 EPCs. We were limited in our sample sizes at a single junction because there was a slow increase in membrane leak produced by the large current we had to pass at holding potentials of −100 mV or −140 mV. Sets of about 200 maximized our sample size with acceptable stationarity of the EPCs collected. Data were taken only from junctions with MEPP frequencies less than 2/sec, so that the probability of a spontaneous release being taken as an evoked release was low ($p < 0.02$).

The data were analyzed as follows. The first 50 points on each record were used to calculate the baseline and the standard deviation of the points about the baseline. In records of EPCs there often was a small, artifactual displacement of the baseline as a part of the stimulus artifact. Therefore, the computer was programmed to begin searching for a peak after the end of the stimulus. From the peak, the program moved backward point by point until a value was encountered that was less than the mean of the baseline + 1 SD. This point was taken as the beginning of the EPC. The points from the peak onward in time were smoothed by a routine that is equivalent to fitting with a fourth-degree polynomial (Stavitsky and Golay, 1964). Starting from the peak, the smoothed points were tested until one was encountered that was less than the mean + 1 SD. This was taken as the end of the EPC. A straight line was then calculated between the points at the beginning and the end of the EPC. This line was taken as the baseline. The peak amplitude of the EPC above this new baseline was recorded, as was the half-time for the decay of the EPC and the time integral of the EPC. The data were viewed before acceptance; the EPC was rejected if it appeared to have a MEPC on its falling phase. It was accepted even if two quantal events could be distinguished on the rising phase, since this probably represented asynchronous evoked releases. The number of times the stimulus was not followed by an EPC (the "zeros") was used to calculate the probability of quantal release, m_o, by

$$m_o = -1n(\text{zeros/total number of stimuli})$$

(del Castillo and Katz, 1954a).

The amplitude, area, and half-time of decay of the MEPCs was calculated using the same subroutine. However, the point to start to look for a peak was indicated by a light pen. MEPCs that appeared to include more than one quanta were rejected.

Usually, neither the amplitude nor integral of the EPCs or the MEPCs appeared to fit a normal distribution function; the curves usually were skewed to the right. Therefore, the Kolmogorov-Smirnov statistic was used as a distribution free test for differences between the distributions of the EPCs and the MEPCs. The cumulative distribution functions were plotted, and the maximum difference between the two curves, D_n, was measured. The probability p, that the D_n between the two curves could by produced by chance is approximated by

$$p = 2 \cdot \exp(-2(D_n n))^2$$

where n is given by

$$n = ((n_1 + n_2)/(n_1 n_2))^{0.5}$$

Results

Figure 2 shows examples of the experimental records. Several criteria were set for a successful experiment. The spike component of the stimulus artifact had to end before the beginning of the EPC. Otherwise the measured EPC was distorted by the artifact. In some experiments when the stimulus failed to elicit an EPC, there still was a noticeable inward current. Apparently this depolarization is produced by K^+ released from the motor nerve terminal (Katz and Miledi, 1983). This depolarizing current would add to the EPCs, thereby distorting their size. The K^+ depolarization reaches a maximum at a membrane potential of −140 mV (Katz and Miledi, 1983), which was a level we frequently employed to obtain a favorable signal to noise ratio. Therefore, we carefully examined the traces for indications of this current and did not record from junctions at which it was noticeable. Two data sets that were taken were subsequently rejected when the K^+ current was detected when the tracings were examined later.

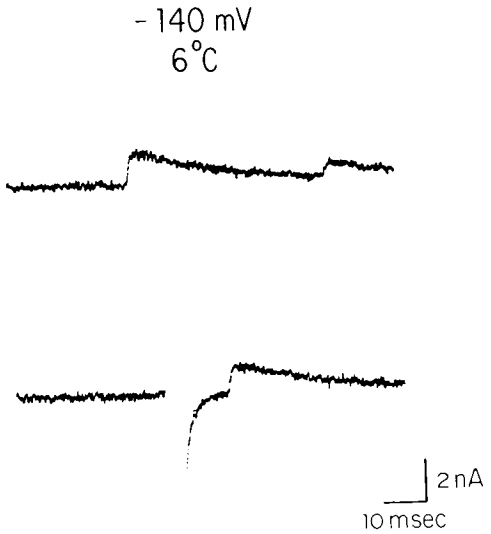

Figure 2. Examples of the data from an experiment at 6°C. The MEPCs are *above*, and the EPC is *below*.

An example of the cumulative distribution functions of the time integrals of the EPCs and the MEPCs from a representative experiments are shown in Figure 3. We decided to use the time integrals for the detailed analysis because they seemed most likely to detect a difference (however, the analysis of the amplitudes led to the same conclusions). The results of the comparison of the time integrals of the EPCs and the MEPCs are summarized in Table I. In each case the comparison is with all of the EPCs recorded. In the experiments the mean quantal output, m_o was kept low, but, nevertheless, in every example a few of the EPCs should, by chance, have been generated by the release of more than one quantum. Two of the 17 examples (I and M) were outside the 95% confidence limit.

Example I is also notable for having the highest m_o of the series. The cumulative distribution functions for example I are shown in Figure 4; the distribution of the EPCs is larger than that of the MEPCs. Therefore, the difference between the two curves might well be produced by multiquantal release. There were 72 EPCs following 200 stimuli. By the Poisson distribution 17 of the releases would be expected to be multiquantal. Figure 4 also shows the cumulative distribution of the EPCs redrawn after the 17 largest had been eliminated from the series. This brings the two curves closer together; they are no longer significantly different ($p = 0.12$). This suggests that the apparent significant difference between the MEPCs and the EPCs in this example is probably an artifact resulting from working with too high a probability of release.

This leaves only one example (M) in which the two curves differ significantly. In this experiment the mean quantal release was low, m_o was 0.1, so the difference is unlikely to be caused by the inclusion of too many multiquantal releases. Perhaps this is simply the occasional deviation expected by chance.

Table I shows that in 12 of the 17 examples the mean of the EPCs was greater than the mean of the MEPCs. This might suggest that there is a trend in the data, but because the difference is relatively small we failed to detect a significant difference. Of course this is possible, but it is important to recall that in all instances some multiquantal releases should by chance occur in the EPC series and a small effect of subsynaptic K^+ accumulation should also be present, so it could be surprising if there were not a tendency toward higher mean EPC values.

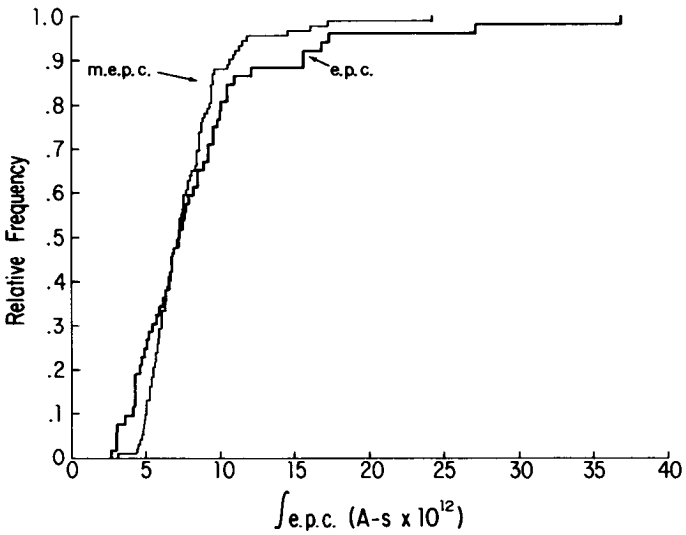

Figure 3. An example of the cumulative distribution plots of the time integrals of the MEPCs and the EPCs. This is example L in Table I.

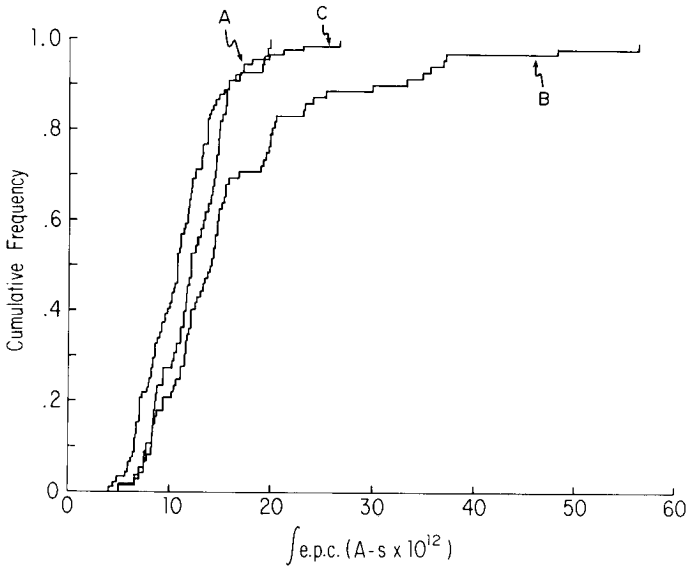

Figure 4. The cumulative distribution plots for the MEPCs (*A*) and the EPCs (*B*) from example I. As discussed in the text, by the Poisson distribution the EPCs would be expected to include 17 multiquantal releases. When the 17 largest EPCs are removed from the set the result is distribution (*C*), which is not significantly different from the distribution of the MEPCs.

TABLE I

Comparison of evoked and spontaneous quanta

Endplates were voltage clamped at potential E_m in low Ca^{2+}-high Mg^{2+} Ringer. The integrals of spontaneously released quanta (MEPCs) and of the current following stimulation of the nerve (EPCs) were measured. From the number of nerve stimulations that did not elicit a response, the mean quantal output, m_0, was calculated. The probability that the differences between the size of the EPCs and the MEPCs could occur by chance are shown in column 9 (p).

Example	E_m	Temperature	EPC	No.	m_o	MEPC	No.	p
	mV	°C	*pA-sec*			*pA-sec*		
A	−140	7	35.5 ± 2.8	51	0.29	29.3 ± 0.8	188	0.77
B	−100	5	22.2 ± 1.8	39	0.27	17.5 ± 3.2	15	0.20
C	−140	7	27.9 ± 1.4	43	0.14	24.0 ± 0.6	150	0.19
D	−100	5	18.6 ± 1.5	24	0.14	21.5 ± 1.2	68	0.37
E	−140	7	19.5 ± 1.4	33	0.17	18.7 ± 1.0	80	0.99
F	−140	6	23.9 ± 1.7	36	0.25	25.9 ± 1.0	162	0.51
G	−140	6	19.2 ± 1.2	33	0.22	21.9 ± 1.1	88	0.66
H	−97	11	9.3 ± 3.3	25	0.09	6.9 ± 0.2	91	0.10
I	−140	11	16.2 ± 1.1	72	0.50	10.8 ± 0.4	91	0.01
J	−140	11	21.1 ± 8.9	22	0.08	24.1 ± 0.9	58	0.12
K	−140	11	21.0 ± 1.5	35	0.12	19.0 ± 0.7	98	0.60
L	−100	11	8.5 ± 0.8	52	0.21	7.4 ± 0.3	98	0.36
M	−100	15	12.4 ± 0.9	29	0.10	9.6 ± 0.3	104	0.02
N	−100	15	8.9 ± 0.9	35	0.13	7.2 ± 0.4	36	0.46
O	−100	15	13.4 ± 1.0	23	0.08	12.2 ± 0.3	91	>0.99
P	−100	15	6.7 ± 0.7	20	0.07	6.2 ± 0.2	104	>0.99
Q	−100	15	8.4 ± 0.6	47	0.17	8.1 ± 0.4	90	0.06

The most important point shown by the data in Table I is that there is no indication of any change in the relation between MEPC and EPC size with changes in temperature.

Discussion

The conclusion is that there is no evidence for any shift in the relation between EPCs and MEPCs as the temperature is changed. This argues that the MEPC and the EPC are produced by the same mechanism.

Cohen and Van der Kloot (1983) studied the relationship between MEPC amplitudes and the depolarization produced by set doses of iontophoretically applied ACh at temperatures between 6 and 15°C. We concluded that there probably is little change in the quantity of ACh per quantum over this temperature range, which favors the vesicle hypothesis. Unfortunately, there was an error in the calculation of one point on our summary illustration (Fig. 4, Cohen and Van der Kloot, 1983). The point at 6°C should be at −21% rather than at −2%. We concluded from these experiments that the Q_{10} for the ACh per quantum of ACh was less than 1/1.28; this conclusion should now be revised to less than 1/1.65. Considering the variance in the MEPCs in a population and the technical difficulties with reproducible iontophoresis, we think it likely that the amount of ACh per quantum in a MEPC is unchanged over this temperature range. The present experiments extend this conclusion to the EPCs, since they are changed by temperature to the same extent as the EPCs. The spontaneous and evoked quanta also appear to be of similar size; however, variations of less than 15% would have been difficult to detect with our statistical approach given the sample size and variance of the EPC and MEPC integral distributions.

Elmquist and Quastel (1965) showed that in the rat neuromuscular junction treated with hemicholinium, an inhibitor of ACh synthesis, there was a parallel decrease in the size of the MEPPs and of the quantal units in the EPPs. Their results strongly support the concept that spontaneous and evoked quantal release share the same mechanism. However, it appears that in certain circumstances larger quanta are released spontaneously than by stimulation (Dennis and Miledi, 1974; Bennett et al., 1976). Also remaining unexplained is the more rapid incorporation of false transmitter into quanta released by stimulation than into those released spontaneously (Large and Rang, 1978a, b) and the simultaneous appearance of small and "normal-sized" spontaneous and evoked quanta at the same endplate (Kriebel et al., 1976).

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