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QUELLING OF SPONTANEOUS TRANSMITTER RELEASE BY NERVE IMPULSES IN LOW EXTRACELLULAR CALCIUM SOLUTIONS

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SUMMARY

1. The effect of nerve stimulation on spontaneous transmitter release was studied at the frog neuromuscular synapse which was bathed in a solution containing very low extracellular calcium concentration. Conventional methods for intracellular and extracellular recording were used and the pattern of quantal liberation following the nerve stimulus was determined.

2. Stimulation of the motor nerve (at rates between 0·09 and 2 Hz) caused a reduction in the frequency of the miniature e.p.p.s in comparison to the pre-stimulation values.

3. The mean distribution of the time of occurrence of the miniature e.p.p.s during the interstimulus period showed periodic oscillations.

4. The quelling effect of nerve stimulation on transmitter release is explained by the hypothesis that at low $[Ca]_o$ a reversed electrochemical gradient for calcium occurs and nerve stimulation causes an increased calcium conductance leading to calcium efflux which in turn temporarily reduces $[Ca]_i$ and transmitter release.

INTRODUCTION

Neurotransmitter liberation from motor nerve terminals occurs in preformed packets named quanta (Fatt & Katz, 1951 1952; Katz, 1969). At rest, these quanta are released spontaneously and can be detected at the post-synaptic muscle membrane as miniature end-plate potentials (m.e.p.p.s). When an action potential arrives at the terminal, the probability of release of these quanta is greatly increased and the outpour of transmitter leads to the formation of an end-plate potential (e.p.p.) (del Castillo & Katz, 1954). This increased probability of quantal release is markedly dependent upon the extracellular calcium concentration, $[Ca]_o$ (del Castillo & Stark, 1952; Jenkinson, 1957; Katz & Miledi, 1965*a*; Dodge & Rahamimoff, 1967; Hubbard, Jones & Landau, 1968*a*). The depolarization brought about by the action potential

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increases the conductance of the nerve membrane to calcium ions, which enter the nerve along their electrochemical gradient (Katz & Miledi, 1967; Baker, Hodgkin & Ridgway, 1971; Llinas & Nicholson, 1975). This entry of calcium ions presumably triggers transmitter release. When this calcium entry is blocked, transmitter liberation is decreased (cf. Baker, 1972). It could therefore be assumed that transmitter release is a function of the intracellular calcium concentration, $[Ca]_i$. This assumption is strengthened by the observation that calcium ions applied internally at the squid giant synapse terminal augment the background rate of transmitter release (Miledi, 1973).

The present work is based on the above mentioned experimental results and on two assumptions. First, it was assumed that normal spontaneous transmitter release reflects the resting free Ca concentration inside the motor nerve terminal. Secondly, it was assumed that the increase in calcium conductance, produced by the action potential, occurs also in extracellular media containing very low calcium concentrations. If these two assumptions are correct, then it is expected that at very low $[Ca]_o$ a reversed electrochemical gradient for calcium will occur due to the arrival of an action potential at the motor nerve terminals, leading to an augmented efflux of calcium and a decrease in quantal release. The aim of the present study was to examine this hypothesis.

Some of the results presented here have been reported in brief previously (Rotshenker, Erulkar & Rahamimoff, 1976).

METHODS

The experiments were done on the sartorius nerve–muscle preparation of the frog (*Rana pipiens* and *Rana ridibunda*).

The preparation was dissected in standard Ringer solution (116 mM-NaCl, 2·0 mM-KCl, 1·8 mM-$CaCl_2$, adjusted to pH 7·0) at room temperature (19–23 °C). $[Ca]_o$ was lowered in two stages by isotonic substitution for Na: first, the preparation was bathed in a medium containing 0·3 mM-$CaCl_2$ and 1 mM-$MgCl_2$ Ringer solution and recording was undertaken to locate a functioning synapse, whereupon a series of e.p.p.s and m.e.p.p.s were recorded. Once this was done, the $[Ca]_o$ was lowered again by perfusing the preparation with 1 mM-EGTA (EGTA = ethyleneglycol-bis-(β-amino-ethyl ether) N,N′-tetra-acetic acid) 1 mM-Mg Ringer solution with no added Ca. (The perfusion rate was approximately 3 ml./min, and the bath volume was 5 ml.). This effectively decreases the Ca concentration levels to less than 10^{-8} M (Bjerrum, Schwartzenbach & Sillen, 1971; Hubbard, Jones & Landau, 1968*b*; Miledi & Thies, 1971) and causes a reversed electrochemical gradient during the arrival of the action potential. The e.p.p.s were abolished and typically the m.e.p.p.s were slightly reduced in frequency. The preparation was allowed to equilibrate in this solution for a minimum period of twenty min during which time several changes of solution were made.

The nerve was stimulated by 0·1 msec pulses at rates of 0·09–2 Hz. Intracellular and extracellular focal recordings were done by means of 3 M-KCl electrodes. In each experiment between 300 and 3600 responses were recorded on a Hewlett-Packard FM tape recorder and then photographed from a 502A Tektronix oscilloscope.

In order to determine the time-course of the release following each stimulus the 'moving-bin' method was used (Rahamimoff & Yaari, 1973). This method consists essentially of aligning all m.e.p.p.s in an array at their appropriate times of occurrence following a stimulus. A bin size of arbitrary duration is then chosen and the frequency within each bin following the stimulus is determined. The origin and end points of each bin are then moved along the time domain by some Δ bin time period and the procedure repeated. (An example of this procedure is illustrated in Fig. 2.) The method was programmed in FORTRAN IV and MACRO ASSEMBLER and run on the PDP 15/78 computer of the Hebrew University-Hadassah Medical School.

RESULTS

The basic observation and the method of presentation are illustrated in Figs. 1 and 2. A frog neuromuscular preparation was bathed for approximately thirty min in a Ringer solution containing 1 mM-EGTA and 1 mM-$MgCl_2$ with no added Ca. This effectively reduced $[Ca]_o$ to less than 10^{-8} M and when the action potential arrived at the nerve terminal a reversed electrochemical gradient for calcium ions was

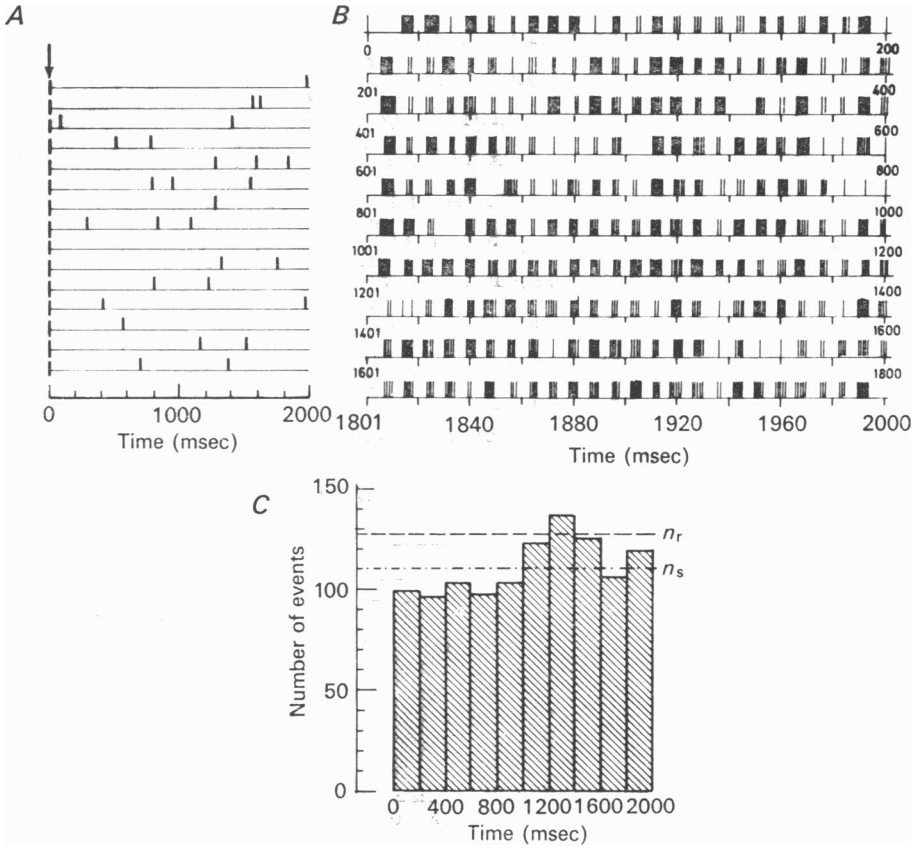

Fig. 1. The effect of nerve stimulation on quantal transmitter release under reversed electrochemical gradient for Ca. The motor nerve was stimulated once every 2 sec and the preparation was bathed in a Ringer solution containing 1·0 mM-EGTA, 1·0 mM-$MgCl_2$ and no added Ca.

A: diagrammatic representation of a sample of the records: the arrow indicates the time of the stimulus; since the basic frequency of the m.e.p.p.s is low, no specific pattern can be observed upon examination of the individual records.

B: the time of appearance of all 1107 m.e.p.p.s which followed 458 stimuli; for convenience of representation, the time array was subdivided into ten consecutive lines of 200 msec; the digitalization unit was of 8 msec, and m.e.p.p.s which have the same time of appearance were represented as clusters

C: histogram of the data in *B*; the histogram unit is 200 msec; n_r is the mean number of events expected if the resting frequency prior to stimulation had continued during the stimulation period; n_s is the mean number of events during the stimulation period (see text).

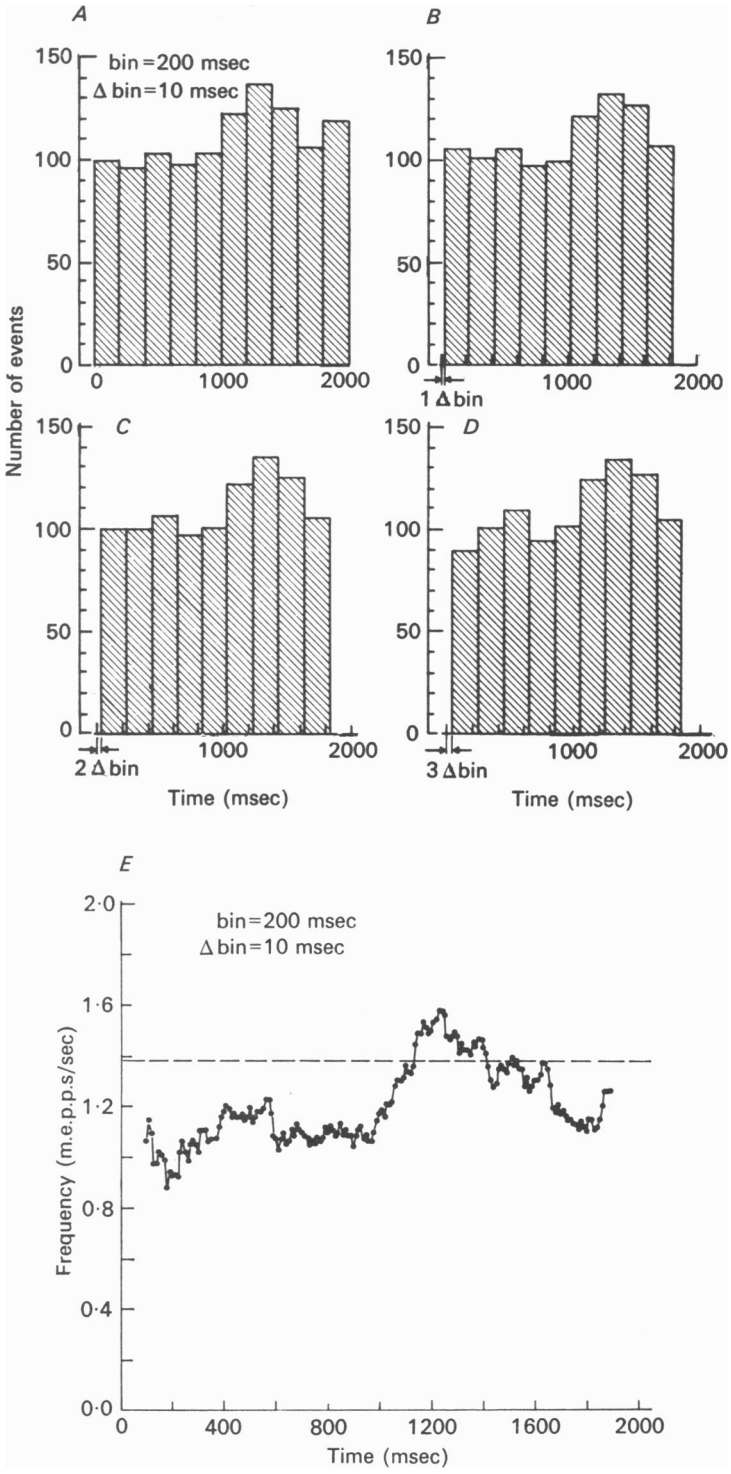

Fig. 2. For legend see facing page.

created (see Discussion). Since under these conditions no evoked e.p.p. is elicited, the effect of the nerve stimulation can be detected only by the frequency of the m.e.p.p.s. However, this frequency is very low in each individual interstimulus period and the effects of many stimuli have to be superimposed in order to estimate the result of the action of the nerve impulse on transmitter release. In Fig. 1*A* sample traces are shown schematically with the appropriate time of appearance of the m.e.p.p.s, following nerve stimulation at a rate of 0·5 Hz. Altogether, 458 stimuli were delivered and the times of appearance of 1107 m.e.p.p.s were superimposed in a single array (Fig. 1*B*). (The digitalization unit in film reading was 8 msec, hence m.e.p.p.s having the same time interval from the stimulus are shown as clusters around the multiple of the digitalization unit.) This array was then subdivided into bins of arbitrarily chosen durations and a histogram constructed. In Fig. 1*C* the bin duration is 200 msec. Already in these histograms it was clear that nerve stimulation in EGTA–Ringer solution was followed by a reduction in m.e.p.p. frequency below resting levels. The resting frequency of the m.e.p.p.s, in the period immediately preceding the nerve stimulation, was 1·39 m.e.p.p.s/sec. If this frequency would have continued during the nerve stimulation period of 916 sec, then 1273 m.e.p.p.s are expected, but only 1107 were observed. In each bin period of 200 msec, the expected number of m.e.p.p.s is 127·3 (n_r in Fig. 1*C*), while the observed average number of m.e.p.p.s in a 200 msec bin is 110·7 (n_s in Fig. 1*C*). Furthermore, the deficiency of m.e.p.p.s was larger in the initial bins after the stimulation in very low $[Ca]_o$.

The presentation of the results in Fig. 1 gives a rather coarse evaluation of the effect. A clearer picture emerges when successive histograms were formed, displaced from each other along the time domain by 'Δ bin' time period (Fig. 2*A–D*). The value of Δ bin was set at 10 msec and three displacements are illustrated. In each case, the calculated frequency was placed at the mid point of the time interval; the curve obtained by this specific manipulation of the data is shown in Fig. 2*E*. Three main features of post-stimulation m.e.p.p. pattern in EGTA–Ringer solution can be seen; first, the average frequency of the m.e.p.p.s was lower compared to that during the resting period preceding stimulation. This occurred in sixteen out of seventeen experiments and the magnitude of this effect varied between 8 and 40 % in different experiments. Secondly, the reduction in the post-stimulus m.e.p.p. frequency in EGTA–Ringer solution was most profound in the first second after the stimulus. Thirdly, the changes in m.e.p.p. frequency were not monotonic but showed periodic oscillations. The latter point was presented to the Physiological Society in brief (Erulkar & Rahamimoff, 1976), and will be described in detail elsewhere.

It should be noted that the phenomena observed in very low $[Ca]_o$ solutions are not stationary with time. The mean frequency of m.e.p.p.s and the degree of

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Fig. 2. A 'moving bin' representation of the results in Fig. 1. A histogram of all available events is constructed with bin width of 200 msec (*A*). The bins were then moved along the time axis by a Δ bin of 10 msec; the resulting histograms after 1 move (*B*), 2 moves (*C*) and 3 moves (*D*) are shown. From the number of events in each bin, the frequency of m.e.p.p.s in each bin was calculated and its value placed in the middle of the bin. The end result is shown in *E*. Since the bin width is 200 msec the first point appears after 100 msec.

depression occasionally decreased with stimulation time; the oscillations changed their periodicity only to a small extent throughout any given experiment (Fig. 3).

The question arises whether these patterns of transmitter release result from the

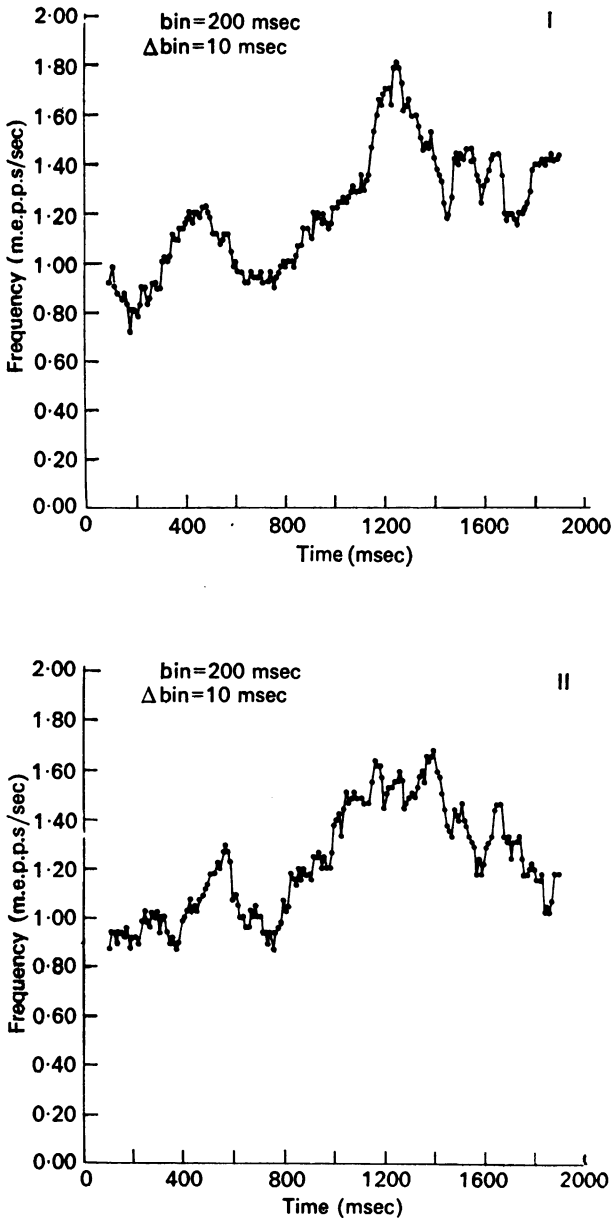

Fig. 3. The variations with time in transmitter release under reverse gradient. The data in Fig. 2 were subdivided into two consecutive time periods of 458 sec. Upper graph is the first and the lower graph is the second time period. Note that although there are differences between the two graphs, both the decrease in m.e.p.p. frequency after the stimulus, and the oscillation are present. The resting frequency prior to the two series was 1·39/sec.

summed activities of many release sites at the terminal or whether they can be detected at each individual release location. This can be tested by the use of extracellular focal recording (Fatt & Katz, 1952; del Castillo & Katz, 1956; Hubbard & Schmidt, 1963; Katz & Miledi, 1965*b*) where the electrode records selectively from a length of a few micrometres of the junction in contrast to the total junctional region recorded from by an intracellularly placed electrode. Fig. 4 is an example of such an experiment; it shows the times of appearance of 200 m.e.p.p.s following 756 nerve

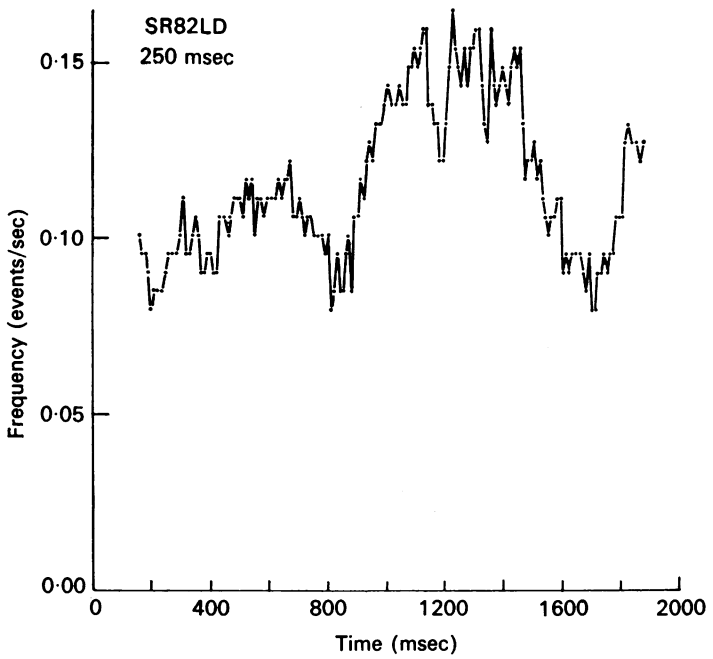

Fig. 4. The suppression of m.e.p.p. frequency by nerve impulses under reverse gradient. Focal extracellular recording. The graph shows the average response to 1241 stimuli. Digitalization unit 10 msec, bin 250 msec. The large number of stimuli was necessary due to the low frequency of m.e.p.p.s.

stimuli at 0·5 Hz. The mean frequency before the stimulation was 0·208/sec and during the stimulation period it was 0·132/sec. It is apparent that also under these conditions the same three phenomena (i.e. depression in transmitter release relative to resting values, uneven distribution of m.e.p.p.s during the interstimulus interval and periodic oscillations in transmitter release) are present.

DISCUSSION

This paper deals with changes in transmitter release induced by low frequency stimulation of the motor nerve in media containing very low $[Ca]_o$. Although such stimulation does not produce an e.p.p., it caused a definite change in the subsequent spontaneous transmitter release. The main experimental observation is the decrease in the frequency of the m.e.p.p.s. As mentioned in the Introduction, the interpretation of such results is based on the notion that quantal release of transmitter is

determined by the concentration of free calcium ions *within* the terminal; if this is the case then transmitter release will be determined by the resting calcium concentration in the terminal, the calcium conductance and the electrochemical gradient across the presynaptic nerve terminal membrane. In order to estimate this gradient, three values are needed: $[Ca]_i$, $[Ca]_o$ and the electrical potential across the presynaptic terminal membrane. None of these parameters has been measured at the frog motor nerve terminal so only approximate estimates can be used from measurements made on other tissues. From suppression potential measurements at the squid giant synapse (Katz & Miledi, 1969) and from light emission studies using the calcium-sensitive protein aequorin (Baker *et al.* 1971; Llinas & Nicholson, 1975) it appears that $[Ca]_i$ is approximately 10^{-7} M. Recent experiments of DiPolo, Requena, Brinley, Mullins, Scarpa & Tiffert (1976) indicate that the intracellular ionized [Ca] in the squid axoplasm is even lower ($0{\cdot}2$–$0{\cdot}5\ 10^{-7}$ M). $[Ca]_o$ depends on the calcium binding properties of EGTA and the contamination of the perfusing fluid with Ca. It should be remembered that the muscle is a huge store and a possible source for Ca. If one takes the Ca dissociation constant given by Bjerrum *et al.* (1971), then $[Ca]_o$ is approximately 4×10^{-9} M (see also Miledi & Thies, 1971). This will give an equilibrium potential for Ca between -30 and -40 mV. Since the resting membrane potential is probably close to -70 mV, then under resting conditions an inward electrochemical gradient is to be expected and only during the depolarization by the action potential would a reverse gradient be established. However, if the ratio Ca_o/Ca_i is smaller than 4×10^{-3} a reverse gradient may occur even at rest.

The simplest explanation for the initial depression of transmitter release is that when the action potential invades the terminal there is a conductance increase to Ca ions which move along their electrochemical gradient and leave the inside of the terminal. This Ca efflux from the terminal effectively decreases $[Ca]_i$ at locations responsible for transmitter release, causing a decrease in the frequency of the m.e.p.p.s. The latter increase in transmitter liberation must reflect a replenishment of Ca at these locations within the terminal and this can only be obtained from internal sources within the terminal, such as the mitochondria (cf. Alnaes & Rahamimoff, 1975) or axoplasmic Ca buffers (Baker & Schlaepfer, 1975; DiPolo *et al.* 1976).

An alternative explanation is that the action potential causes an influx of EGTA across the presynaptic terminal membrane. This would effectively decrease $[Ca]_i$ with a consequent reduction in transmitter release. The later increase in release would result from the removal or dispersion of the EGTA from the terminal. This explanation is less attractive because of the apparently low permeability of EGTA across some biological membranes (Weber, Herz & Reiss, 1966).

Regardless which of these mechanisms actually occurs, the net effect in each case is a decrease in $[Ca]_i$ and a reduction in transmitter release. One puzzling feature of the initial depression of transmitter release is its long-lasting duration which can continue for up to one sec. We have no explanation for this prolonged suppression. It may reflect the nature of the control processes in transmitter release, which seem to impose periodic oscillations (see Erulkar & Rahamimoff, 1976).

In the physiological range of Ca concentrations, the relation between evoked transmitter release and $[Ca]_o$ follows a high-power saturation kinetics (Dodge &

Rahamimoff, 1967; Hubbard *et al.* 1968*a*; Katz & Miledi, 1969; Crawford & Fettiplace, 1971). If $[Ca]_0$ is reduced to less than 0·1 mM by the use of EDTA-Ca buffers, the relation converts to first power (Crawford, 1974). This conversion has been interpreted by assuming two sources of Ca for release: the amount that enters due to the action potential and the 'resting' Ca inside the nerve terminal. As the contribution from the first source diminishes, the relative importance of the second increases, leading to a less steep dependence on $[Ca]_0$ (see also Miledi & Thies, 1971; Rahamimoff & Alnaes, 1973). The present results show that the action potential may even have a negative contribution to the resting Ca and cause a suppression of transmitter release, if a reverse electrochemical gradient is created. This suggests that at least part of the resting release of neurotransmitter quanta is determined by the free Ca inside the nerve terminal; when these Ca levels are decreased, the resting release is diminished.

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