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TETANIC AND POST-TETANIC RISE IN FREQUENCY OF MINIATURE END-PLATE POTENTIALS IN LOW-CALCIUM SOLUTIONS

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SUMMARY

1. Miniature end-plate potentials (min.e.p.p.s) were recorded intracellularly from frog neuromuscular junctions.

2. The 'phasic' release of transmitter which is directly related to nerve impulses was suppressed by withdrawal of Ca from the external medium plus addition of Mg.

3. Under these conditions, min.e.p.p.s continued to be discharged even when EGTA was added, although in this case min.e.p.p. frequency appeared to decrease to about half the rate in normal Ringer.

4. Tetanic stimulation of the nerve approximately doubled the rate of min.e.p.p.s even in Ca-free solutions with EGTA added.

5. The tetanic increase in frequency was greater without EGTA and greater still with some Ca added. Therefore, it is concluded that the tetanic rise in min.e.p.p. frequency can occur even in the absence of the immediate 'phasic' release of transmitter normally induced by nerve impulses; and that the magnitude of the increase is related to Ca concentration.

A possible relation between 'phasic' and 'residual' effects of nerve impulses is described.

INTRODUCTION

At the neuromuscular junction, the discharge of miniature end-plate potentials (min.e.p.p.s) is accelerated for a period following tetanic stimulation of the nerve (Brooks, 1956; Liley, 1956; Hubbard, 1963; Braun, Schmidt & Zimmerman, 1966). A similar increase in frequency of miniature synaptic potentials has been observed also at neuro-neuronal synapses (Miledi, 1967). The mechanism of this increase in quantal leakage of transmitter from nerve terminals remains unknown. To gain some insight into the problem it was thought of interest to see if the increase in min.e.p.p.

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frequency was still observed when the release of transmitter by nerve impulses had been abolished by withdrawing Ca ions from the external medium. In these conditions it was found (Miledi & Thies, 1967) that, although much reduced, an increase in frequency still occurred.

Normally the motor nerve impulse is followed, after a brief delay, by an intense surge of quantal transmitter release (Katz & Miledi, 1965*b*). After withdrawal of external Ca this 'phasic' release is reduced to practically zero, while there is little or no diminution in the spontaneous background discharge of min.e.p.p.s. It will be shown that, although the phasic response to a nerve impulse vanishes in low-Ca media, there is still a cumulative effect during prolonged impulse bombardment: the min.e.p.p. frequency gradually rises, and slowly subsides after the end of stimulation over a period which is closely related to the normal period of post-tetanic potentiation (Feng, 1941). A possible relation between 'phasic' and 'residual' effects of motor impulses will be considered in the Discussion.

METHODS

The experiments were made on frog sartorius muscles at room temperature (19–23° C), using techniques described in detail by Katz & Miledi (1965*a* to *c*). Ringer's solution was made up with British Drug Houses Analar grade salts and distilled water; it routinely contained (mM): NaCl 111·6, KCl 2·0, $NaHCO_3$ 2·4, $MgCl_2$ 1·8, and neostigmine methylsulphate (10^{-6} g/ml.). 'Ca-deficient' Ringer was prepared by omitting $CaCl_2$.

Superficial neuromuscular junctions were located microscopically, and min.e.p.p.s were recorded with intracellular electrodes, often simultaneously at two junctions. Oscilloscope records were photographed on continuously moving film before, during, and after application of supramaximal stimuli to the nerve at frequencies of 50 or 100/sec for 19–21 sec. During each period of tetanic stimulation a fraction of the stimuli ($\frac{1}{2}$—$\frac{1}{10}$) triggered 20 msec sweeps on a second oscilloscope that were photographed with a second camera. In a few experiments extracellular micro-electrodes filled with 0·5 M-$CaCl_2$ were used to increase local $[Ca^{2+}]_o$ and thereby permit transmitter release to single test stimuli before or after tetanic stimulation. The same electrodes or other micro-electrodes filled with Indium, often served to record action potentials from non-myelinated nerve terminals (Katz & Miledi, 1965*a*).

Although the probability of 'phasic' transmitter release in response to a single impulse is very low in Ca-deficient Ringer (Katz & Miledi, 1968), this probability is increased during rapid repetitive stimulation (del Castillo & Katz, 1954*b*). Therefore, the Mg concentration was raised in some experiments to 10 or 20 mM to minimize such phasic responses, osmolarity being maintained by reduction of NaCl to $\frac{7}{8}$ or $\frac{3}{4}$ of its usual concentration. In other experiments, phasic release was permitted by adding 0·2 or 0·45 mM Ca to Mg-enriched, Ca-deficient Ringer. Finally, in many experiments $[Ca^{2+}]$ was reduced to less than 10^{-8} M by addition to Ca-deficient Ringer of 1 mM ethyleneglycol bis (amino-ethylether)-*N*,*N*′-tetra-acetic acid (EGTA), obtained from General Chemical Co. (Judex). This was used in preference to EDTA, because the binding constant for Mg by EGTA is much less than by EDTA (Sillen & Martell, 1964). Magnesium concentration was raised to 2 mM to ensure conduction of nerve impulses in solutions with such low [Ca].

The [Ca] in Ringer without added $CaCl_2$ was determined by the method of Kerr (1960) and was found to be between 0·01 and 0·02 mM. This is more than the 0·004 mM measured by Curtis (1963) for choline Ringer, probably due to an impurity of about 0·008 mM-Ca in the NaCl (Frankenhaeuser, 1957), as well as the use of distilled water that was not de-ionized. In addition, contamination of $MgCl_2$ could contribute as much as 0·005 mM-Ca when 20 mM-$MgCl_2$ was used in the Ringer. When Ca-deficient Ringer was allowed to bathe sartorius muscles for 6–16 hr, Ca concentrations of 0·05–0·06 mM were measured in the Ringer bath (*ca.* 15 ml.) demonstrating leakage of Ca from muscles. Since more than half of muscle [Ca] is lost per hour (Shanes & Bianchi, 1959; Bianchi, 1961), and solutions bathing muscles were routinely changed at hourly intervals, the maximum [Ca] in 'Ca-deficient' Ringer for these experiments will be taken as 0·03 mM.

Addition of 1 mM-EGTA to Ca-deficient Ringer containing traces of Ca from impurities reduces [Ca] about 10,000-fold, depending upon pH. Stock solutions of 0·1 M-EGTA (Na salt) were prepared by addition of 1 m-mole EGTA to 10 ml. 0·2 M-NaOH and adjusting pH to just above 7 with HCl solution. If one uses a stability constant of $7·6 \times 10^6$ for pH 7·1 (Portzehl, Caldwell & Rüegg, 1964; Sillen & Martell, 1964), then 0·03 mM Ca and 1 mM-EGTA result in an estimated $[Ca^{2+}]$ of 4×10^{-9} M.

RESULTS

Min.e.p.p.s and tetanic stimulation. The frequency of min.e.p.p. discharge increases progressively during the period of tetanic stimulation and declines slowly afterwards. This is illustrated in Fig. 1, which shows intracellular records obtained simultaneously from two fibres in a muscle in Ca-deficient Ringer with 20 mM-Mg. Stimulation at 100/sec began in the middle of the upper records (interrupted lines in Fig. 1*A*). The rate of min.e.p.p.s was already increased in the first 2 sec after the onset of stimulation. During the last 2 sec of stimulation, shown in the first half of Fig. 1*B*, min.e.p.p. frequency was 15–20 times higher than before stimulation. During the 2 sec shown after cessation of stimulation the frequency was still more than 10 times above the control. These particular values were the highest observed among all the fibres tested in Mg-enriched Ca-deficient Ringer.

The time course of this frequency change during and after tetanic stimulation is plotted for another fibre in Fig. 2. Min.e.p.p. frequency is shown on the ordinates as the ratio of mean frequency during blocks of 4 sec to the mean control value during a 1 min period before stimulation. In this fibre the frequency increased continuously during stimulation, up to between 3 and 4 times the control rate at the end of the tetanus, a typical effect in Mg-enriched, Ca-deficient Ringer. The frequency declined to near control values during the next few minutes.

Phasic release of transmitter. The immediate release of transmitter in response to a single nerve impulse is abolished by the omission of Ca from Ringer or the addition of 10 or 20 mM-Mg (del Castillo & Katz, 1954*a*). However, during tetanic stimulation transmitter release is facilitated, and

it could be that some of the min.e.p.p. activity observed *during* the period of stimulation is due to direct release by the nerve impulse. To distinguish between phasic and residual effects of nerve impulses during the period of stimulation, the timing of min.e.p.p.s relative to the impulses was examined in the following manner.

The onset of a normal end-plate potential was determined by stimulating the nerve during focal application of Ca to the end-plate. As in previous experiments (Katz & Miledi, 1965*a* to *c*), after the arrival of the impulse

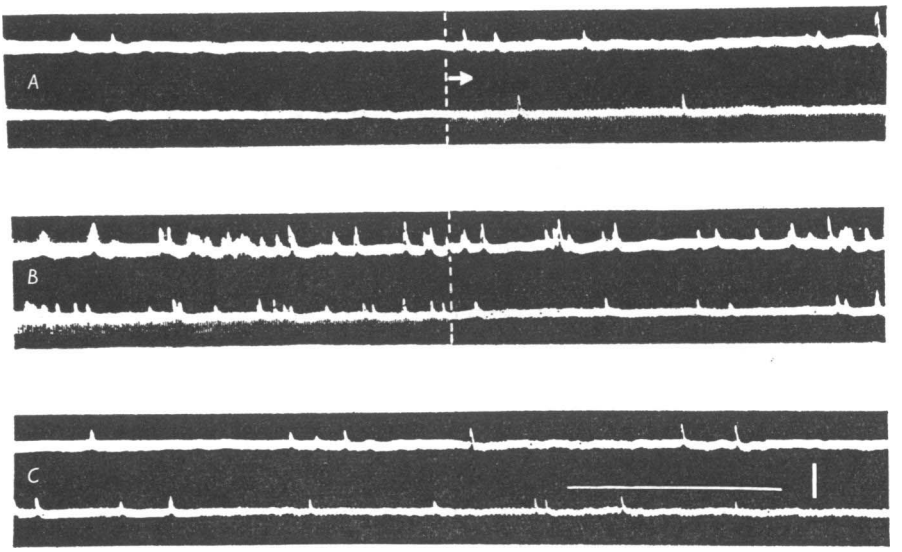

Fig. 1. Tetanic increase of min.e.p.p. frequency in Ca-deficient Ringer with 20 mM-Mg. Two fibres recorded simultaneously. *A*, *B* and *C* 4 sec sampling periods. *A*: 2 sec before and 2 sec after the start of stimulation at 100/sec at interrupted lines. *B*: the last 2 sec of the 19 sec period of stimulation and 2 sec after stimulation stopped. *C*: 120 sec after the end of stimulation. 1 mV and 1 sec calibrations for all records.

at the terminal, there was a brief period during which no transmitter release was observed. This time plus the impulse conduction time, from the site of stimulation to the site of recording was approximately 3 msec in the experiment illustrated in Fig. 3. Since practically all the phasic release of transmitter is accomplished within the first 5 msec after the arrival of the nerve impulse, we may take it that min.e.p.p.s appearing in the first 3 msec after a nerve stimulus is given to the nerve, represent the background min.e.p.p. activity. Any min.e.p.p.s in excess of this amount would then correspond to quantal release due to the phasic action of the nerve impulse.

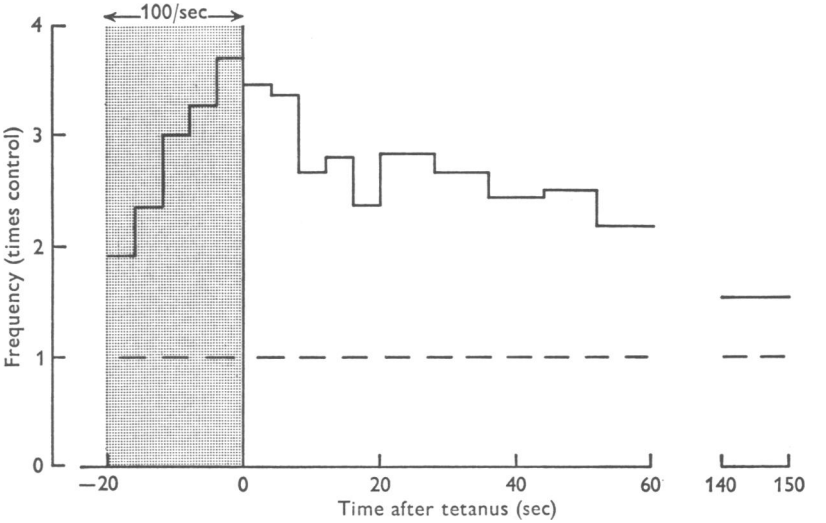

Fig. 2. Time course of increase of min.e.p.p. frequency in Ca-deficient Ringer with 10 mM-Mg. Abscissa: time after cessation of stimulation at 100/sec for 20 sec; note break between 60 and 140 sec. Ordinate: ratio of frequency of min.e.p.p. during 4 sec intervals to control frequency of 6·5/sec during 1 min before tetanus. Stippled area during the period of stimulation.

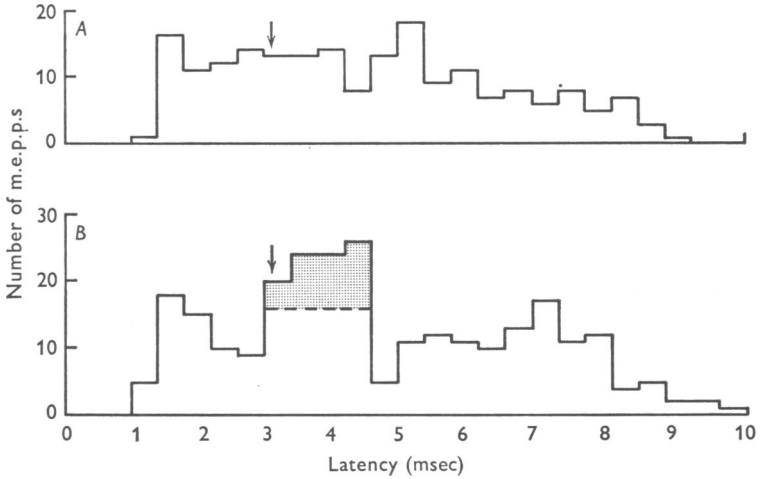

Fig. 3. Latencies between stimuli and min.e.p.p.s during tetanic stimulation at 100/sec in Ca-deficient Ringer with 10 mM-Mg. *A* and *B*: distributions of latencies throughout two successive periods of stimulation; abscissa: number of min.e.p.p.s in 0·4 msec classes. Downward arrows at latency end-plate potential in response to nerve stimulus during focal Ca application. Stippled area of *B* for excess min.e.p.p.s that probably represent phasic responses to nerve impulses.

For instance, the distribution of min.e.p.p. latencies during two successive periods of tetanic stimulation are shown in Fig. 3. During the first period (Fig. 3*A*, data from same trial as in Fig. 2) approximately the same number of min.e.p.p.s occurred at all intervals between 1 and 8 msec. (Stimulus artifacts, film perforations, etc., distorted the measurements between 0–1 and 8–10 msec). During the second period of stimulation 46 min later (Fig. 3*B*), 'excess' min.e.p.p.s occurred at latencies of 3·0–4·6 msec, just where end-plate potentials would be expected to occur. This is evidence that perhaps some thirty of the 267 min.e.p.p.s recorded during 1030 stimuli were in fact phasic responses, representing a release of single packets of transmitter in approximately 3 % of the stimuli.

A further indication that some phasic release occurs during tetanic stimulation in high-Mg Ca-deficient Ringer, is afforded by the fact that in some fibres the min.e.p.p. frequency fell abruptly to less than half the facilitated value as soon as the stimulation was stopped. Such an immediate lowering of frequency is seen in Fig. 1*B* (lower trace) and again indicates that some of the min.e.p.p.s recorded just before the end of the stimulation are due to phasic release by nerve impulses. In view of this, it was decided to further reduce the external Ca concentration to less than 10^{-8} M by chelation with 1 mM-EGTA, retaining 2 mM-Mg.

Min.e.p.p.s in Ca-free solutions. It has been claimed (Elmqvist & Feldman, 1965) that min.e.p.p.s completely disappear from muscles kept in Ca-deficient Ringer with a chelating agent added. However, as already reported (Miledi & Thies, 1967) and recently confirmed (Hubbard, Jones & Landau, 1968) this is not so, and min.e.p.p.s can be recorded after many hours in Ca-free solutions. In fact, we have found min.e.p.p.s even in a muscle which had been continuously perfused for 16 hr with Ca-deficient Ringer with 1 mM-EGTA. In these solutions the frequency of miniature potentials usually appeared to be reduced to about 30–45% of the control. But, it was found that when 1 mM-EGTA or EDTA was added to the Ca-deficient Ringer the min.e.p.p. amplitude fell to about 50–75 % of normal, and it could be that some of the frequency reduction is only apparent and due to failure to detect the smaller min.e.p.p.s.

Before proceeding to test the effect of tetanic stimulation, it was necessary to show that the low Ca concentrations achieved with EGTA did not completely impair the functioning of the neuromuscular junction. Extracellular focal recording from unmyelinated nerve endings showed that even after several hours in Ca-free Ringer with 1 mM-EGTA the nerve impulses continued to invade the terminals, although some failure of invasion can occur with prolonged tetanic stimulation. Furthermore, iontophoretic application of Ca to a synaptic spot quickly restored transmitter release, indicating both that the nerve impulse invaded the terminal

and that the nerve membrane can rapidly bind Ca even in the presence of the chelating agent.

Some cumulative rise in min.e.p.p. frequency still occurred during tetanic stimulation in Ringer with less than 10^{-8} M-Ca present. The example illustrated in Fig. 4 shows that during stimulation at 100/sec for 20 sec min.e.p.p. frequency rose in irregular fashion from a control rate of less than 1/sec to about twice that rate. After stimulation ceased, the min.e.p.p. discharge remained at the higher level for about 1 min and approached the initial values 2 min after stimulation.

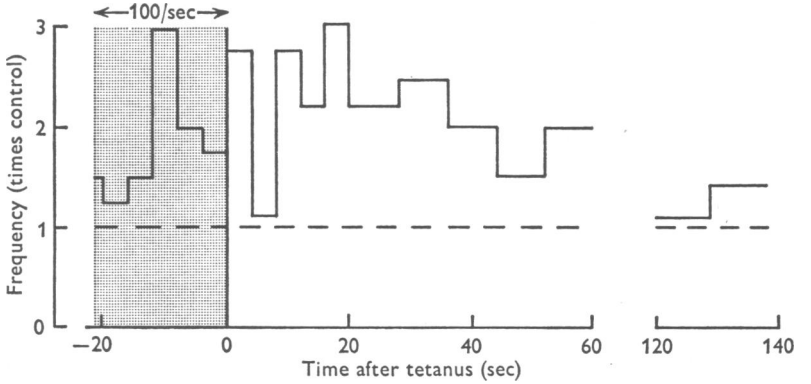

Fig. 4. Increase of min.e.p.p. frequency in Ca-deficient Ringer with 2 mM-Mg and 1 mM-EGTA. Abscissa: time after cessation of stimulation at 100/sec for 21 sec; note break between 60 and 120 sec. Ordinate: ratio of frequency of min.e.p.p.s during 4 sec intervals to control frequency of 0·9/sec during 2 min before tetanus. Stippled area during stimulation period.

Thus there was no doubt that the rise in min.e.p.p. frequency still occurred when EGTA was added to Ca-deficient Ringer, in conditions when no phasic release was detected. But it was interesting that the rise was smaller, indicating that the magnitude of the effect is related to the $[Ca]_o$ concentration. This relationship is shown in Fig. 5, where the increase in frequency is plotted against the ratio of $[Ca]_o/[Mg]_o$ concentrations. The use of this ratio rather than Ca alone made it easier to compare experiments with various concentrations of Mg.

When EGTA was used ($[Ca]_o/[Mg]_o = 2 \times 10^{-6}$) the tetanic increase in min.e.p.p. frequency was, on the average, about 1·7 (range 1·1–2·05). With higher concentrations of Ca the rise in frequency was more pronounced. But since in some end-plates there was some phasic release, it could be thought that the increased effect is not due directly to the increase in Ca but to some increased metabolic state associated with phasic release of transmitter. When this is taken into account it can be seen that even in those fibres where no phasic release could be detected (circles in Fig. 5)

the tetanic effect on min.e.p.p. frequency increased with Ca-concentration. It is interesting to note that if we restrict ourselves to fibres with no phasic release during the tetanus, a more than 1000-fold increase in $[Ca]_o$ concentration barely doubled the tetanic rise in min.e.p.p. rate.

Fig. 5 also includes the results of two experiments (arrows) with 1·8 mM-Ca, which is the normal concentration in Ringer. In these cases the control min.e.p.p. rate was determined, and the micro-electrode was then

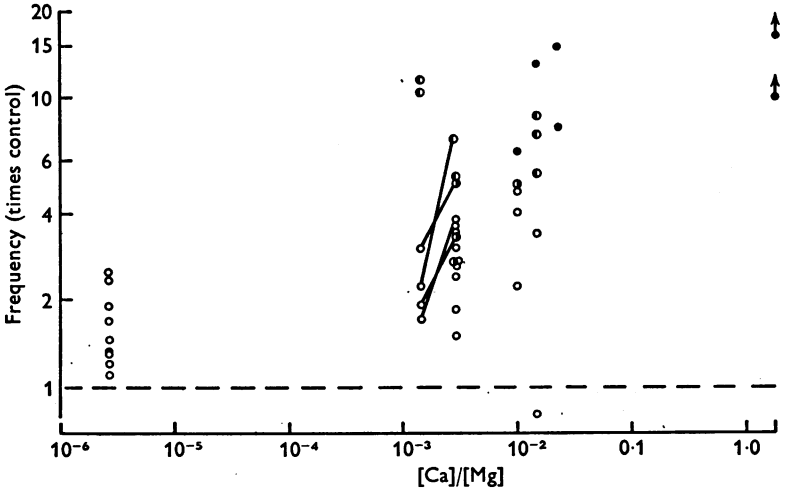

Fig. 5. Effect of [Ca]/[Mg] on magnitude of tetanic increase of min.e.p.p. frequency. Abscissa: log of [Ca]/[Mg]. Ordinate: log of ratio of min.e.p.p. frequencies during the first sec after tetanus compared with 20 sec before tetanus, except for 20 sec after tetanus in fibres with [Ca]/[Mg] less than 10^{-5}, where no min.e.p.p.s occurred in the first sec after tetanus in four of nine trials. ● with upward arrow: fibres in normal Ringer. ●: fibres with responses (e.p.p.s) to more than 7 % of stimuli during tetanus, estimated from extra min.e.p.p.s at 3–5 msec after stimuli (cf. Fig. 3). ◑: responses to 1–3 % of stimuli during tetanus, estimated from latency measurements. ◐: some e.p.p.s in response to stimuli, estimated by more than 50 % decrease in min.e.p.p. frequency when stimulation stopped. ○: no response to stimuli, since min.e.p.p. frequency decreased by less than 20 % when stimulation stopped. Lines connect successive trials in the same fibres at altered [Ca]/[Mg].

withdrawn to avoid damage to the fibres during the vigorous contraction which accompanied tetanic stimulation. At the end of the stimulation a few seconds were allowed for the muscle to relax, and the electrode was reinserted. In the two fibres shown in Fig. 5, when the min.e.p.p. frequencies were measured 25 and 33 sec after the end of the tetanus, they were 10 and 16 times the control values. The maximal effect must have been larger, since min.e.p.p. frequency declines after the tetanus.

DISCUSSION

The question arises whether (*a*) the immediate, phasic, release by a nerve impulse, and (*b*) the cumulative residual effects of impulses on frequency of min.e.p.p.s (together with 'facilitation' and 'potentiation' phenomena) are due to quite separate processes, or whether they can be explained on a unitary basis. It is sometimes argued that the 'late' phenomena, i.e. post-tetanic potentiation and associated frequency rise of min.e.p.p.s, must arise in an entirely different way from the 'early' release, because they may show a delayed rising phase. This, however, is more apparent than real, as a phase of depression commonly intervenes, at normal Ca concentrations, during a prolonged tetanus. Although other schemes are possible, it is still conceivable that transmitter release, facilitation, tetanic and post-tetanic potentiation, and prolonged rise of min.e.p.p. frequency, could all be a direct consequence of a single process, namely initial inward movement of Ca ions followed by gradual inactivation or removal of 'active Ca' (Ca*) from the release site at the inner surface of the axon membrane.

As already pointed out by Katz & Miledi (1968) if one is to explain both transmitter release and subsequent facilitation on such a unitary basis, it is necessary to assume that the removal of Ca* follows non-linear kinetics, e.g. that the rate of elimination of Ca* is proportional to the nth power of Ca* ($n > 1$). In this case, the decline of Ca* would become disproportionately slower, and its effects more cumulative, as the concentration falls.

The situation could be simulated by adding a series of exponentials with different time constants, but for the present we will simplify it even further by dividing the temporal course of Ca* into two blocks, a strong but very brief phasic component of amplitude A which lasts only about 1 msec, and a weak residual component of intensity B whose rate of decay is so slow that it accumulates linearly during the 20 sec tetanus.

The following assumptions are made regarding the relation between external calcium concentration $[Ca]_o$, the *relative* concentration of active Ca*, transmitter release (m), and frequency of min.e.p.p.s (f):

(1) The initial 'phasic' peak of Ca* immediately following the arrival of the impulse, i.e. the value of A, is proportional to the $[Ca]_o$, and (like B) remains the same for every impulse throughout the tetanus. The residue, however, i.e. the value of B, is not proportional to $[Ca]_o$, and diminishes only slightly as $[Ca]_o$ is reduced. (This is a simplified substitute for a non-linear rate equation.)

(2) Transmitter release (i.e. the number of quantal packets m released by the impulse) varies with Ca*, and therefore with $[Ca]_o$. During the early

'phasic' release, the relation between m and $[Ca]_o$ is of the Michaelis–Menten type proposed by Dodge & Rahamimoff (1967, Fig. 1, ignoring presence of Mg); that is, it obeys a 4th power law at low levels of release (roughly, with $m \leqslant 0{\cdot}1$ and $[Ca]_o \leqslant 10^{-4}$ M), but its slope greatly diminishes at higher concentrations and release rates. We are making the further assumption that an identical relation holds, *at all times*, between rate of transmitter release and the *relative* concentration of Ca*. Thus, a 4th power relation between Ca* and m will be assumed, except for the high phasic values of Ca* in Table 1*B*, where calculation of m has been omitted. Concentration of Ca* will be expressed relative to that attained at the end of a 20 sec tetanus in 3×10^{-5} M-Ca (Table 1).

TABLE 1. Comparison of phasic and residual transmitter release at different Ca concentrations

A	3×10^{-5} M-Ca		
	Phasic	$m_1 = 2{\cdot}5 \times 10^{-3}$	$Ca_1^* = 0{\cdot}7$
		$m_{2000} = \mathbf{8 \times 10^{-2}}$	$Ca_{2000}^* = 1{\cdot}7$
	Residue	$m_1 = 6 \times 10^{-16}$	$Ca_1^* = 5 \times 10^{-4}$
		$m_{2000} = \mathbf{10^{-2}}$	$Ca_{2000}^* = 1$
B	$1{\cdot}8 \times 10^{-3}$ M-Ca (ignoring any intervening 'depression' of release)		
	Phasic		$Ca_1^* = 42$
			$Ca_{2000}^* = 43{\cdot}8$
	Residue	$m_1 = 6 \times 10^{-15}$	$Ca_1^* = 9 \times 10^{-4}$
		$m_{2000} = \mathbf{10^{-1}}$	$Ca_{2000}^* = 1{\cdot}8$
C	'4×10^{-9}' M-Ca		
	Phasic	$m_1 = (8 \times 10^{-19})$	$(Ca_1^* = 10^{-4})$
		$m_{2000} = (2 \times 10^{-3})$	$(Ca_{2000}^* = 0{\cdot}67)$
	Residue	$m_1 = 1{\cdot}3 \times 10^{-16}$	$Ca_1^* = 3{\cdot}3 \times 10^{-4}$
		$m_{2000} = \mathbf{2 \times 10^{-3}}$	$Ca_{2000}^* = 0{\cdot}67$

(3) Transmitter release can be expressed in two different but equivalent ways: as a rise in the frequency of min.e.p.p.s ('Δf'), or as a quantal number ('m'). The value of 'm' customarily refers only to *phasic* release, i.e. to the number of quantal packets released immediately after each impulse, within a brief period occupying approx. 1 msec at 20° C. This corresponds to a phasic increase in the frequency of min.e.p.p.s ('Δf') of approx. 1000 'm'/sec. The relation 'm' = 'Δf' $\times 10^{-3}$ sec will be used for the purpose of comparing phasic and residual transmitter release.

With these assumptions, the values in Table 1 have been calculated from the data shown in heavy type (which are the typical results obtained at three different $[Ca]_o$ concentrations). For example, consider the experiments with 3×10^{-5} M Ca (Table 1*A*). There was initially no detectable phasic release, but during the tetanus (2000 impulses at 100/sec) a few units gradually began to appear during the phasic release periods, the

final value of m being somewhat less than 10^{-1} (8×10^{-2}). Also, the frequency of min.e.p.p.s rose to about 10/sec, equivalent to $m = 10^{-2}$.

Hence, if we designate the cumulative residue of Ca* at the end of 2000 impulses (i.e. $2000B$) as unity concentration, then the residue of a single impulse (B) was 5×10^{-4}, incapable of raising the value of m by any detectable amount ($10^{-2} \times 625 \times 10^{-16}$). From the phasic release by the *last* impulse ($m = 8 \times 10^{-2}$), we calculate that the value of A (phasic rise of Ca*) amounted to 0·7 ($2000\,B + A = 1 + A$, and $(1+A)^4 = 8 \times 10^{-2}/10^{-2}$). The first impulse raised the value of m to only $(0{\cdot}7/1{\cdot}7)^4 \times 8 \times 10^{-2} = 2{\cdot}5 \times 10^{-3}$, which would have required long periods of testing and been extremely difficult to detect above a basic release rate of nearly half this value. Thus we calculate that the cumulative effect of 2000 impulses can produce 'potentiation', i.e. gradually facilitate a small phasic release and an increase in min.e.p.p. frequency, in spite of the fact that each impulse adds a residue which is less than 1/1000 of the 'subliminal' phasic peak of Ca*.

With normal $[Ca]_o$ (Table 1*B*), the effect of the tetanus was to raise the frequency of min.e.p.p.s to about 100/sec (i.e. $m \rightarrow 10^{-1}$). Using the same scale for Ca* concentration, the latter would rise to 1·8 during the 2000 impulses. The residue is therefore only slightly larger than in 3×10^{-5} M-$[Ca]_o$, and, relative to the phasic entry and formation of Ca*, which is assumed to be proportional to $[Ca]_o$, it is greatly reduced.

In the EGTA experiments ($[Ca]_o$ 4×10^{-9} M; Table 1*C*), the cumulative effect of 2000 impulses was to raise the frequency to about 2/sec ($m = 2 \times 10^{-3}$), equivalent to a calculated Ca* of 0·67. If one tries to derive the phasic values of m, from the Dodge & Rahamimoff 4th power law, one finds that at this very low Ca_o concentration the phasic effects are smaller than the residues, which is of course inconsistent with the assumption of a single, 'monotonic' process of Ca entry and subsequent elimination. Thus, either there are two separate, successive processes of Ca* formation and transmitter release (of which only the first obeys the 4th power law at low Ca_o concentrations), or the effective value of $[Ca]_o$ at the membrane surface is greater than the value of 4×10^{-9} M calculated from stability constants. How high would $[Ca]_o$ have to be in order to satisfy the present postulates? The answer is: high enough to give a phasic m_1 of at least $1{\cdot}3 \times 10^{-16}$. Thus the minimum Ca_o concentration would be $3 \times 10^{-5} \times \sqrt[4]{\{(1{\cdot}3 \times 10^{-16})/(2{\cdot}5 \times 10^{-3})\}}$ which is approximately $1{\cdot}5 \times 10^{-8}$ M. This seems quite feasible, and it would not be very surprising if $[Ca]_o$ at the cell surface remains even somewhat higher than this.

We conclude that with certain assumptions it is possible to maintain the hypothesis that a single rise and fall of Ca* may underly the phasic as well as the cumulative phenomena observed in these experiments. The required assumptions are (*a*) that the ratio $(A+B)/B$ (where A is phasic Ca* and B

is Ca* residue due to each impulse) falls from a very high value to near unity, as $[Ca]_o$ is reduced; (*b*) that the 'effective' value of $[Ca]_o$ does not drop below about 2×10^{-8} M, even in the presence of 1 mM-EGTA; (*c*) that the 4th power relation between $[Ca]_o$ and m, although it has been shown to apply to the phasic release at low $[Ca]_o$, cannot be extended to the long-term cumulative effects. Incidentally, this may explain why such a relation does not fit, when the effect of $[Ca]_o$ on transmitter release is measured by the maintained frequency rise in high K solution (Gage & Quastel, 1966).

Finally, the question remains whether there is a basic spontaneous rate of release, completely independent of Ca*. It has been shown here that such spontaneous release still occurs after many hours in Ca-deficient solutions to which a chelating agent had been added. Nevertheless, the release may depend on the formation of an irreducible amount of Ca* derived from local Ca stores.

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