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A STUDY OF TETANIC AND POST-TETANIC POTENTIATION OF MINIATURE END-PLATE POTENTIALS AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. The involvement of calcium, sodium, potassium and magnesium in tetanic and post-tetanic potentiation of miniature end-plate potential frequency was examined at the frog neuromuscular junction using conventional electrophysiological techniques.

2. Tetanic potentiation is larger in calcium containing solutions, than in solutions which generate reversed electrochemical gradient for calcium during nerve activity.

3. Tetanic potentiation increases with stimulation frequency and duration, under both inward and reversed electrochemical gradient for calcium conditions. This indicates that factors, other than calcium entry, participate in tetanic potentiation.

4. Addition of the potassium conductance blocking agent, 3-aminopyridine (5 mM), increases tetanic potentiation in calcium containing media, while depressing it under reversed calcium gradient.

5. Electronic depolarization of the nerve terminal in tetrodotoxin-containing Ringer solution, produces tetanic potentiation under inward gradient, but fails to do so under reversed gradient. This indicates that the entry of sodium ions participates in the generation of tetanic potentiation.

6. Addition of magnesium ions suppresses tetanic potentiation in calcium containing solution, but increases tetanic potentiation under reversed gradient.

7. The results are explained by the hypothesis that calcium entry and intracellular calcium translocation participate in the generation of tetanic potentiation.

8. Both the fast and the slow components (augmentation and potentiation respectively) of post-tetanic potentiation increase in duration, with increase in the tetanic stimulation rate.

9. The decay of post-tetanic potentiation increases: when $[Ca]_o$ is elevated by ionophoretic application during the decay phase only, when ouabain is present in the medium or when $[Mg]_o$ is elevated. These findings suggest that calcium, sodium and possibly magnesium take part in post-tetanic potentiation.

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INTRODUCTION

The nervous system typically conveys messages by several action potentials within a short period of time. Although the action potentials within the sequence have a similar amplitude, the resulting synaptic potentials vary in size according to their temporal relations. The main effect of this frequency coding is on the presynaptic nerve terminal where the rate of activation determines the mean number of neurotransmitter quanta liberated.

A high rate of nerve activation produces changes in transmitter release during the stimulation period. At low quantal contents the main effect is of potentiation (tetanic potentiation). The potentiation persists for periods of seconds to minutes after the cessation of the high frequency stimulation and is named post-tetanic potentiation. Magleby & Zengel (1976) have shown that it consists of at least two phases – augmentation and potentiation. The finding that the frequency of the spontaneous miniature end plate potentials (m.e.p.p.s) resembles that of the end-plate potential amplitude (e.p.p.) (Rotshenker, Erulkar & Rahamimoff, 1976; Erulkar & Rahamimoff, 1978) permits the use of more drastic changes in the ionic environment in the study of the ionic basis of potentiation.

Earlier studies have shown that calcium ions play an important role in potentiation (Rosenthal, 1969; Weinreich, 1971). However, calcium ions cannot be the only cause for potentiation, since Miledi & Thies (1967, 1971) and Hurlbut, Longenecker & Mauro (1971) demonstrated that tetanic and post-tetanic potentiation appear also in the virtual absence of calcium ions in the external medium. This raises the possibility that other ions involved in neuronal activity may participate in the generation of tetanic and post-tetanic potentiation. The present work consists of two parts: in Part II we examine the possible involvement of sodium, potassium and magnesium ions in the high frequency modulation of transmitter release at the frog neuromuscular junction, while Part I describes the effects of the rates of nerve terminal activity on the parameters of potentiation. Some of the results were reported previously in brief (Lev-Tov, Erulkar & Rahamimoff, 1977; Rahamimoff, Erulkar, Lev-Tov & Meiri, 1978; Lev-Tov & Rahamimoff, 1978, 1979).

METHODS

(1) *Preparation*

Experiments were performed on the sartorius nerve muscle preparation of the frog (*Rana ridibunda* and *Rana pipiens*).

(2) *Solutions*

The preparations were dissected at room temperature (19–23 °C) in standard frog Ringer (115·6 mM-NaCl, 2 mM-KCl and 1·8 mM-$CaCl_2$). Calcium was lowered and magnesium was added (1–10 mM) by isotonic substitution for sodium. The pH was adjusted to 6·9–7·1 before using. Reversed calcium gradient conditions during nerve activation were obtained by addition of 1mM-EGTA to frog Ringer from which calcium was omitted (see Erulkar, Rahamimoff, Rotshenker, 1978).

(3) *Stimulation*

(*a*) Tetanic trains of square pulses (duration of 0·02 msec each) were delivered to the motor nerve at various frequencies and durations. The intervals between trains were 5–30 min.

(*b*) After addition of tetrodotoxin (TTX) the transmitter release was evoked by electrotonic depolarization of the nerve (Katz & Miledi, 1967*a*). The nerve was carefully dissected towards its fine branches and then drawn into a suction electrode and sealed by Vaseline. The synaptic activity was recorded from fibres adjacent to the entry of the nerve; the stimulation pulse was prolonged to 1 msec to obtain a sufficient electrotonic depolarization upon nerve stimulation. This produced frequently large artefacts of stimulation, which precluded the possibility of estimating m.e.p.p. frequency reliably during the tetanic stimulation. In such experiments, only the post-tetanic frequency was estimated.

(4) *Recording*

Synaptic activity was recorded by conventional methods for intracellular recording. Focal extracellular recording was done by a calcium pipette (Katz & Miledi, 1965*a*, *b*; Dodge, Miledi & Rahamimoff, 1969; Barrett, Barrett, Martin & Rahamimoff, 1974).

Some of the results were recorded on Hewlett Packard FM tape recorder and then filmed. The digitalized results were analyzed by a PDP 15/78 (see Erulkar *et al.* 1978; Erulkar & Rahamimoff, 1978).

RESULTS

PART I: CALCIUM AND THE KINETIC PARAMETERS OF TETANIC AND POST-TETANIC POTENTIATION

Tetanic potentiation

During tetanic nerve stimulation, there is an increase in m.e.p.p. frequency (Del Castillo & Katz, 1954; Hubbard, 1963; Weinreich, 1971; Miledi & Thies, 1971; Kamenskaya, Elmqvist & Thesleff, 1975) which depends on the stimulation rate and duration (Fig. 1). Table 1 summarizes the result of sixty-eight experiments performed on twelve end-plates, where the extracellular $[Ca]_o$ was 50 μM. Three points are obvious. First, the normalized end tetanic frequency (the ratio of the end tetanic frequency/resting frequency) increases with the rate of nerve stimulation (Fig. 2*A* and 2*B*) and with the duration of the stimulation; secondly, the total number of quanta liberated by the intensive nerve stimulation at this low $[Ca]_o$ is rather small and it does not exceed the number of quanta released by a single nerve impulse under normal $[Ca]_o$ (see Katz, 1962, 1969); hence the possibility of depletion of transmitter quanta is excluded and one can study the ionic basis of potentiation without this complication. Third, the effectiveness of nerve stimulation increases with stimulation frequency and duration, much more than expected from a simple algebraic addition of the number of stimuli delivered to the motor nerve. For example, stimulation of the motor nerve at a rate of 50 Hz for 10 sec, produced a barely noticeable effect, and only 6 quanta were liberated more than in a comparable period during rest (on the average 0·012 quanta per nerve impulse). Stimulation at the same rate, but for 80 sec, produced 376 extra quanta (on the average 0·094 quanta/impulse). Therefore, there is a cumulative effect of nerve stimulation in tetanic potentiation. In part *C* of the Table an equal number of stimuli were delivered at different rates and durations; at the highest rate of stimulation (100 Hz)

each impulse is about 14 times more effective than at the lowest (10 Hz). Frequencies below 10 Hz were usually ineffective.

It should be noted that two sources of variability exist in the experimental results. First, there is a substantial variability in the magnitude of the effect among different

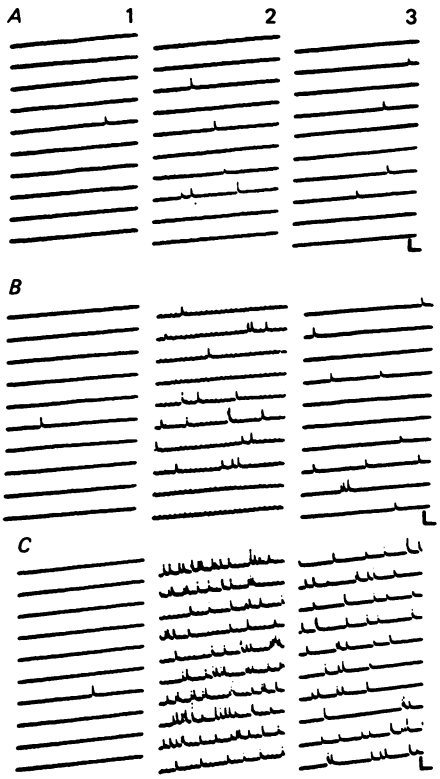

Fig. 1. Tetanic and post-tetanic potentiation of miniature end plate potential frequency (f) at different stimulation rates and durations. Sample records of prestimulation f (f_r) are shown in column 1, of end tetanic stimulation f (f_s) in column 2 and the initial poststimulation f (f_{ps}) in column 3. *A*, response to a tetanic stimulation of 25 Hz for 40 sec ($f_r = 0{\cdot}2$ sec^{-1}, $f_s = 1{\cdot}2$ sec^{-1} and $f_{ps} = 0{\cdot}8$ sec^{-1}). *B*, response to a tetanic stimulation of 50 Hz for 40 sec ($f_r = 0{\cdot}18$ sec^{-1}, $f_s = 4{\cdot}2$ sec^{-1} and $f_{ps} = 2{\cdot}4$ sec^{-1}). *C*, response to a tetanic stimulation of 50 Hz for 80 sec ($f_r = 0{\cdot}16$ sec^{-1}, $f_s = 22{\cdot}6$ sec^{-1} and $f_{ps} = 12{\cdot}0$ sec^{-1}). Intracellular recording in a Ringer solution containing 50 μM-calcium chloride, 2 mM-magnesiun chloride. Calibration: vertical bar, 1 mV; horizontal bar, 50 msec.

end-plates (compare Fig. 2*A* with Fig. 2*B*); therefore, the geometric averages are also shown in Table 1. In addition, there is a progressively larger effect of the same tetanus at any given end-plate, when the interval between the tetani is shorter than 20 min (Fig. 2*C*), showing that the cumulative effect at the end of the tetanus persists for many minutes after it.

TABLE 1. The effect of duration and frequency of stimulation on tetanic and post-tetanic potentiation at the frog neuromuscular junction

Frequency of stimulation (Hz)	Duration (sec)	Number of stimuli	Number of experiments	Excess quanta (Q)	$\frac{Q}{\text{impulse}}$	Normalized end tetanic frequency ± S.E. of mean	Normalized end tetanic frequency GA*	Normalized initial post-tetanic frequency ± S.E. of mean	Normalized initial post-tetanic frequency GA*	τ_A (sec)	τ_p (sec)
					A duration						
50	10	500	6	6·11 ± 3·06	0·012	3·6 ± 0·922	3·06	1·75 ± 0·43	—	—	16·58 ± 7·36
50	20	1000	6	14·87 ± 3·69	0·015	8·53 ± 0·62	5·9	2·41 ± 0·62	1·95	—	37·5 ± 8·2
50	40	2000	24	71·6 ± 10·37	0·036	11·04 ± 1·66	10·91	8·02 ± 1·06	6·45	2·26 ± 0·82	80·5 ± 8·65
50	80	4000	3	376·3 ± 177·5	0·094	66·66 ± 38·2	37·14	32·26 ± 22·5	16·35	9·8 ± 5·16	129·5 ± 5·15
					B frequency						
10	40	400	7	3·4 ± 0·72	0·0085	1·5 ± 0·21	1·36	1·36 ± 0·39	—	—	—
20	40	800	5	13 ± 4·72	0·01625	4·04 ± 0·28	5·06	2·86 ± 1·39	2·04	—	14·67 ± 7·07
40	40	1600	5	71·54 ± 30·1	0·0447	8·92 ± 1·92	10·65	6·4 ± 1·2	5·7	0·62 ± 0·22	43·2 ± 12·09
50	40	2000	24	71·6 ± 10·37	0·036	11·04 ± 1·66	10·91	8·02 ± 1·06	6·45	2·26 ± 0·82	80·5 ± 8·65
100	40	4000	4	1105 ± 524·85	0·276	141·95 ± 61·05	94·71	48·45 ± 21·59	31·37	9·29 ± 2·05	163·05 ± 31·74
					C equal number of stimuli						
10	200	2000	3	31·05 ± 2·77	0·0155	2·36 ± 1·57	1·48	3·06 ± 0·85	—	—	50·06 ± 8·02
50	40	2000	24	71·6 ± 10·37	0·036	11·04 ± 1·66	10·91	8·02 ± 1·06	6·45	2·26 ± 0·82	80·5 ± 8·65
100	20	2000	5	439 ± 279	0·219	183·8 ± 162	37·75	18·36 ± 8·89	12·84	5·32 ± 2·01	132·72 ± 19·9

* GA = geometric average.

Post-tetanic potentiation

In calcium containing media, when an inward going electrochemical gradient for calcium exists, the frequency of the miniature end plate potentials decreases after the end of the tetanic stimulation to levels lower than the end-tetanic frequency. In eighty-two experiments (from which sixty-eight are shown in Table 1) the average frequency in the first 5 sec after the stimulation was 29·53 % of the end

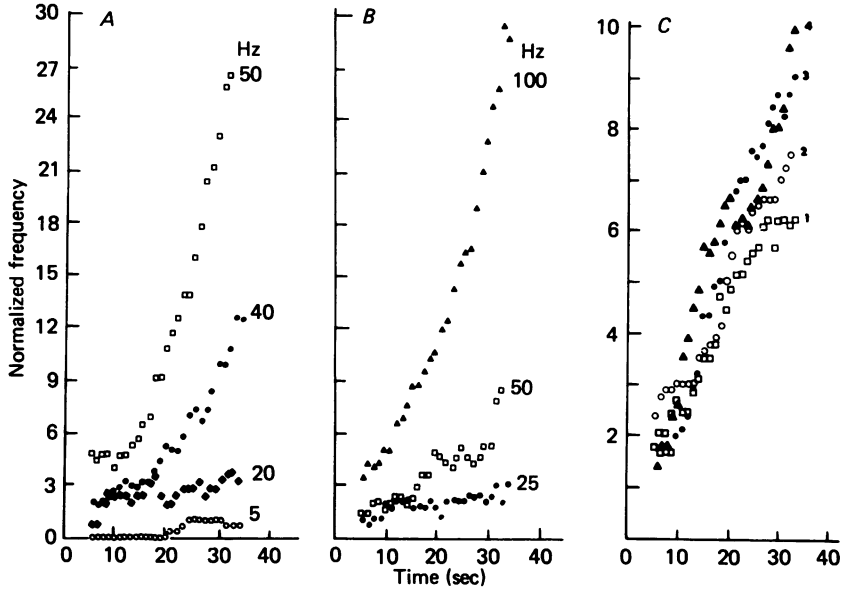

Fig. 2. Tetanic potentiation of m.e.p.p. frequency increases with stimulation rate. *A* and *B* show the response at two different end-plates to stimulation lasting 40 sec and at rates marked on each set of points. The frequency of m.e.p.p. was normalized by dividing by the resting frequency. Note the variability of the response to the same stimulation parameters (50 Hz). *C*, the time course of four sequential tetanic potentiations at the same end-plate (1, 2, 3 and 4) in response to stimulation of 50 Hz for 40 sec. The intervals: 1–2, 515 sec; 2–3, 510 sec; 3–4, 470 sec. Moving bin representation of the data; bin size = 10 sec and Δ bin = 1 sec. Ringer solution containing 50 μM-calcium chloride and 2·0 mM-magnesium chloride.

tetanic *f* (this is contrary to the situation in EGTA containing media – *vide infra*). It has been shown for the post-tetanic decay of the e.p.p. amplitude (Magleby & Zengel, 1976) and for the post-tetanic decay of the m.e.p.p. frequency (Erulkar & Rahamimoff, 1978) that the decay does not follow a single exponential function but can be decomposed into at least two separate exponentials: a fast one named by Magleby & Zengel the augmentation phase and a slower one named potentiation. The contributions of these two phases to the post-tetanic decay in *f* and their time constants depend on the frequency and the duration of the tetanic nerve stimulation. Augmentation usually is prominent only after intensive nerve stimulation and the time constant τ_A increases with the frequency and the duration of the stimulation (Table 1 and Fig. 3). Potentiation appears at lower intensities of stimulation but

otherwise follows the same pattern: its magnitude and time constant increase with the frequency and the duration of the tetanic nerve stimulation (Table 1 and Fig. 3). Phenomenologically, therefore, the general contour of the post-tetanic decay in f is described by the equation

$$f_{ps}(t)=f_A e^{-t/\tau_A}+f_p e^{-t/\tau_P}+f_r.$$

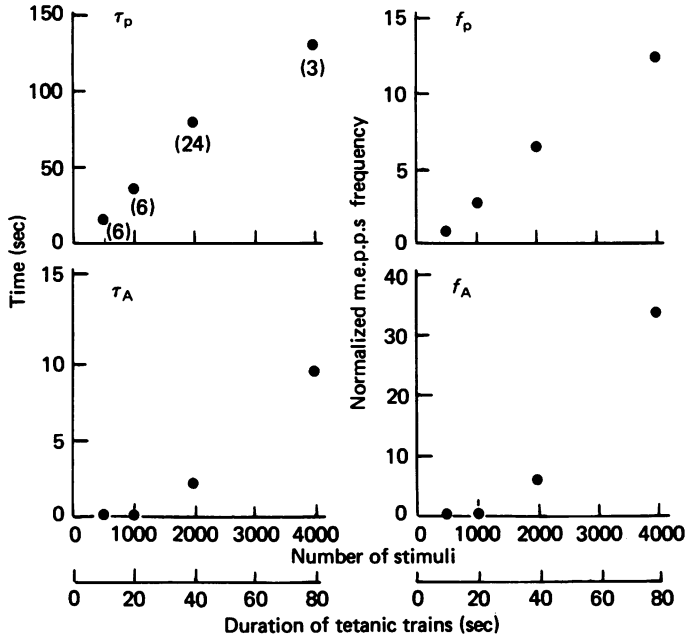

Fig. 3. Post-tetanic potentiation parameters depend on the number of stimuli delivered during the tetanus. Constant stimulation frequency of 50 Hz. f_A = the extrapolated values of augmentation, f_P = the extrapolated values of potentiation, τ_A = the time constant of augmentation, τ_p = the time constant of potentiation. The values were obtained by regression analysis, using the equation in text; arithmetic average of the results. The number of experiments is shown near each point in τ_p. Note the cumulative effect of nerve stimulation.

Where

$f_{ps}(t)$ is the post-tetanic frequency of the m.e.p.p.s at different times after the end of the tetanus,

f_r is the resting frequency of the m.e.p.p.s,

τ_A is the time constant of the decay of the augmentation phase,

τ_p is the time constant of the decay of the potentiation phase and

f_A and f_P are the extrapolated values of the augmentation and the potentiation for $t=0$ (end of the tetanic stimulation).

It should be noted that this equation accounts only for the general shape of the post-tetanic decay, but not for the overriding oscillations in f (Erulkar & Rahamimoff, 1978).

The dependency of the post-tetanic potentiation parameters on the pattern

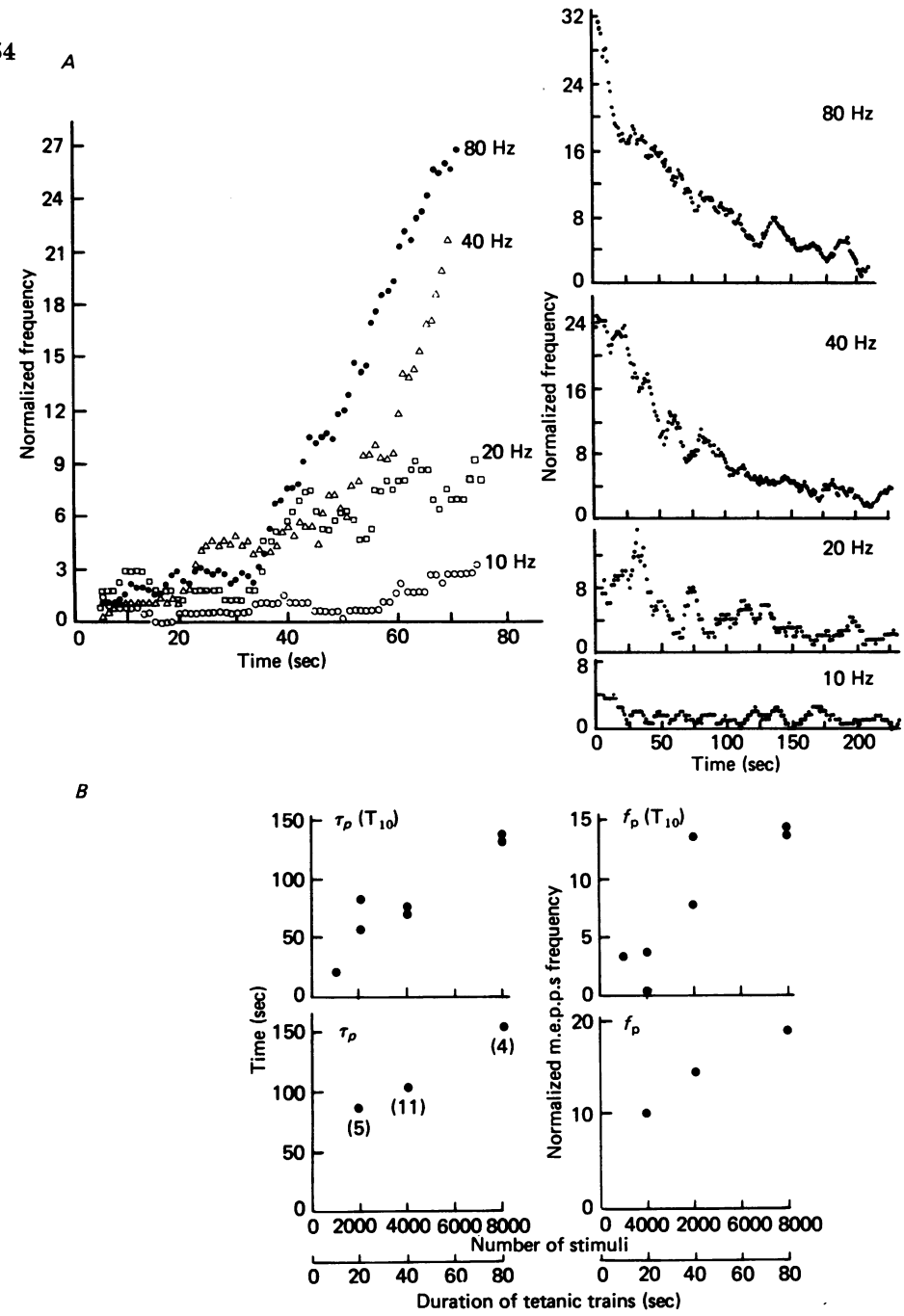

Fig. 4. Tetanic and post-tetanic potentiation in reversed electrochemical gradient for calcium during nerve stimulation: cumulative effects of rate and duration of stimulation. *A*, stimulation rate. *A*, left panel: tetanic potentiation due to stimulation at various rates (denoted on each set of points). *A*, right panel: post-tetanic potentiation due to stimulation at various rates (denoted on each set of points). Constant stimulation duration of 80 sec. Moving bin: bin = 5 sec, Δ bin = 1 sec. *B*, stimulation duration. The effect of the number of stimuli delivered to the motor nerve at constant rate of 100 Hz on post-tetanic potentiation parameters. The upper two panels show the results obtained at a single end-plate (experiment T10). The lower two panels are the arithmetic averages from a number of different experiments (the number of experiments appears in parenthesis). Regression analysis. τ_p = time constant of potentiation, f_p = the value of potentiation extrapolated to the end of the tetanus.

of nerve stimulation is shown in Fig. 3. It is quite clear that the cumulative effect of nerve stimulation on m.e.p.p. frequency, apparent during the tetanic stimulation, persists afterwards for many minutes.

Tetanic and post-tetanic potentiation parameters in reversed calcium electrochemical gradient

Omission of calcium ions from the bathing medium and addition of EGTA, reduce the extracellular calcium concentration to less than 10^{-9} M (Hubbard, Jones & Landau, 1968; Miledi & Thies, 1971). This concentration is lower than the estimated intracellular free [Ca], thus a reversed electrochemical gradient for calcium is generated during the action potential at the presynaptic nerve terminal (see Erulkar *et al.* 1978). Fig. 4 and Table 2 show the main features of the effect of tetanic stimulation on f, under such conditions.

(*a*) Tetanic nerve stimulation produces an increase in f in the absence of $[Ca]_o$, thus extracellular calcium ions are not necessary for tetanic potentiation (but see (*c*)).

(*b*) The tetanic increase in f depends on the frequency of stimulation and its duration (Fig. 4).

(*c*) The effectiveness of the tetanic stimulation is greatly reduced in the absence of $[Ca]_o$. Comparing Table 2 with Table 1 one can see that in the presence of 50 μM-$CaCl_2$, stimulation of 40 Hz for 40 sec produces an average release of 0·0447 quanta/impulse, while in the absence of this small amount of calcium (and in the presence of 1 mM-EGTA) the same stimulation produces on the average only 0·0162 quanta/impulse (or 36·5 %). The difference is even more striking at higher rates of stimulation. Stimulation of 100 Hz for 20 sec causes a release of 0·219 quanta/impulse in the presence of 50 μM-$CaCl_2$ and only 0·0116 quanta/impulse in its absence (5·3 %). So although most of the tetanic potentiation is caused by external calcium ions, there is still a cumulative factor creating a tetanic potentiation in the absence of calcium.

(*d*) After the end of the nerve stimulation in seventy out of seventy-six experiments (92 %) performed at 2·0 mM-$MgCl_2$ a further increase in f was observed. This illustrates that nerve stimulation has a dual effect: it causes an increase in f even under reversed electrochemical gradient for calcium, but it suppresses the full development of the effect. The initial post-tetanic m.e.p.p. frequency in EGTA containing media was lower than in calcium-Ringer solution. This is seen at high rates of stimulation even when the results are normalized to the resting frequency.

(*e*) The post-tetanic increase in m.e.p.p. frequency, typically decays as a single exponential, whose time constant gets larger with an increase in the frequency of stimulation and its duration (Fig. 4). Hence, also in the absence of $[Ca]_o$, the cumulative effect of nerve stimulation persists for minutes after the end of the tetanus.

(*f*) The lack of the augmentation phase in EGTA containing media, raises the obvious question: is that due to the smaller post-tetanic potentiation found in low calcium or is it due directly to the lack of $[Ca]_o$? Again a comparison of the results of Tables 1 and 2 shows that the lack of augmentation is due to the absence of calcium. For example, stimulation of 50 Hz for 40 sec causes a post-tetanic increase of 8·02 times the control ($f_{ps} = 2·83$) and an augmentation with a time constant

TABLE 2. The effect of duration and frequency of stimulation on tetanic and post-tetanic potentiation in reversed calcium gradient conditions at the frog neuromuscular junction

Frequency of stimulation (Hz)	Duration (sec)	Number of stimuli	Number of experiments	Excess quanta (Q)	$\frac{Q}{\text{impulse}}$	Normalized end tetanic frequency		Normalized initial post-tetanic frequency		τ_p (sec)
						± S.E. of mean	GA*	± S.E. of mean	GA*	
					A duration					
100	20	2000	5	23·2 ± 11·21	0·0116	8·12 ± 3·33	5·71	14·76 ± 4·25	9·98	83·34 ± 14·6
100	40	4000	11	71·23 ± 31·82	0·0178	11·78 ± 1·99	11·84	24·3 ± 5·97	17·39	102·04 ± 10·78
100	80	8000	4	233·57 ± 103·8	0·0291	19·2 ± 4·51	17·8	20·15 ± 5·29	18·52	153·17 ± 23·4
					B frequency					
40	40	1600	3	26·06 ± 9·63	0·0162	5·4 ± 1·84	4·6	9·37 ± 2·37	9·01	36·8 ± 12·33
80	40	3200	3	157·63 ± 58·64	0·0492	24·13 ± 6·89	21·89	30·2 ± 9·46	26·4	58·16 ± 12·07
100	40	4000	11	233·57 ± 103·8	0·0291	11·78 ± 1·99	11·84	24·3 ± 5·97	17·39	10·204 ± 10·78

* GA = geometric average.

of 2·26 sec, while in the absence of $[Ca]_o$ stimulation of 100 Hz for 40 seconds produced a larger increase in f (24·3 times the control) (f_{ps} = 5·94), with no sign of the augmentation phase.

The timing of the calcium action in post-tetanic potentiation

The previous section showed that although calcium ions are not an absolute requirement for the development of tetanic and post-tetanic potentiation, they

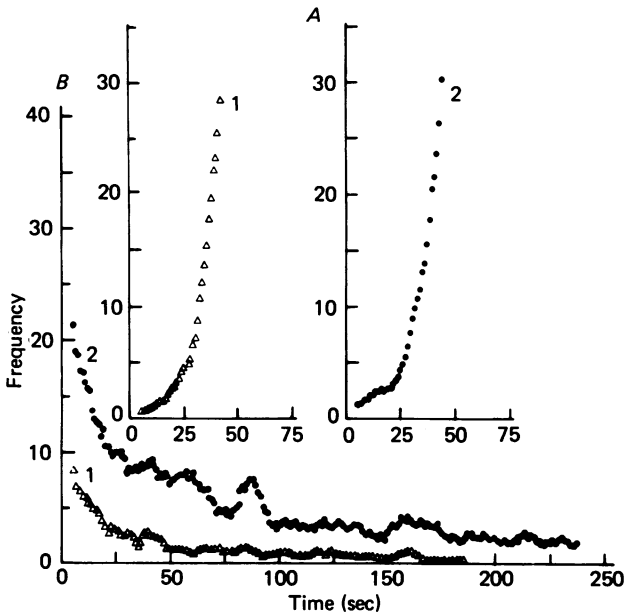

Fig. 5. Post-tetanic calcium ionophoresis. *A*1 and *B*1 are the tetanic and post-tetanic potentiation due to repetitive stimulation at 50 Hz for 40 sec during which high braking current was applied to the calcium pipette (see text), presumably preventing substantial calcium leakage. In experiment 2 the tetanic stimulation was repeated at the same end-plate with a high braking current, producing a similar tetanic response (*A*2); after cessation of the stimulation, the braking current was stopped and calcium was allowed to diffuse from the pipette (*B*2). Note the larger post-tetanic potentiation. Moving bin: bin = 10 sec, Δ bin = 1 sec.

are responsible for a major part of the response. A question remains, however, when do they exert their effect; is it only during the nerve stimulation or also after the cessation of the nerve impulses? For this purpose we used extracellular recording with a calcium filled micropipette. In the control experiment a strong braking current was applied to the calcium pipette, so that no evoked response was observed to low frequency stimulation after the appropriate synaptic delay (cf. Katz & Miledi, 1965*b*, 1968). A tetanic stimulation produced an increase in frequency as shown in Fig. 5*A*1. After the end of the stimulation, f decayed as shown in Fig. 5*B*1. The tetanic stimulation was repeated in Fig. 5*A*2, producing almost identical results; but now after the end of the tetanus, the braking current was stopped and calcium allowed to diffuse from the pipette. One can clearly see that the potentiation

was increased and prolonged (Fig. 5*B*2). In four experiments of this type the mean value of τ_A was 8·92 sec ± 0·84 S.E. of mean with a braking current and it increased to 16·9 sec ± 4·5 S.E. of mean when calcium ions were allowed to diffuse from the pipette; the values for the time constant of potentiation were 97·8 ± 10·77 and 136·62 ± 15·46 sec respectively.

These experiments show that extracellular calcium ions play a predominant role in tetanic potentiation and they have a smaller, but a significant effect in the post-tetanic potentiation. However, even in the virtual absence of $[Ca]_o$, still a substantial fraction of tetanic and post-tetanic potentiation persists. The aim of Part II is to examine which other ions may take part in the potentiation.

PART II: POTASSIUM, SODIUM AND MAGNESIUM IN POTENTIATION

The candidates

It is well known from studies in a large number of excitable tissues that several ions are capable of moving along their electrochemical gradient, when voltage sensitive channels are opened by the action potential. They include sodium which creates the action potential in many tissues by its influx (Hodgkin & Katz, 1949; Hodgkin, Huxley & Katz, 1952; Dodge & Frankenhaeuser, 1958; Hille, 1970), potassium ions that move out of the cell (Hodgkin & Huxley, 1952; Hodgkin *et al.* 1952; Hille, 1970) and magnesium ions that flow inside (Baker & Crawford, 1971; Rojas & Taylor, 1975). In the frog motor nerve terminal these ionic fluxes can presumably cause a substantial change in the cytosolic concentration during high frequency stimulation due to the large surface to volume ratio. The rest of this article deals with the possible contribution of potassium, sodium and magnesium to the development of tetanic potentiation and the time course of post-tetanic potentiation.

The effect of blockade of potassium conductance on potentiation

We used 3-amino pyridine (3-AP) to block potassium conductance (Pelhate & Pichon, 1974; Molgo, Lemeignan & Lechut, 1975; Yeh, Oxford, Wu & Narahashi, 1976; Ulbricht & Wagner, 1976) and to examine its effects on tetanic and post-tetanic potentiation. If the leakage of potassium, and the decrease in $[K]_{in}$ is a major contributor to both processes, it is expected that they will be reduced by 3-AP. But, if the main effect of potassium blockade is on the duration of the action potential and thus on the development of G_{Ca} (Katz & Miledi, 1969), then the effect of the potassium channel blockade will depend on the direction of the calcium gradient.

In the presence of 50 μM-calcium ions in the extracellular medium, a blockade of the voltage sensitive potassium channels by 5 mM-3-AP produced an increase in tetanic potentiation (Fig. 6 and Table 3), which was reversible upon washing (Fig. 6*F*). The mean number of quanta/impulse increased from 0·0403 to 0·298; the effect was dramatic in particular near the end of the stimulation train, when nerve stimulation produced time-locked evoked activity. It should be noted that the experiments were performed in 5 mM-magnesium chloride to prevent excessive time-locked evoked release and eventual muscle twitch. In a reverse electrochemical

gradient for calcium conditions, however, 3-AP had an opposite effect: it reduced the end tetanic frequency to about 50% (from 11·78 to 5·76) and the mean number of quanta per impulse from 0·0178 to 0·00858.

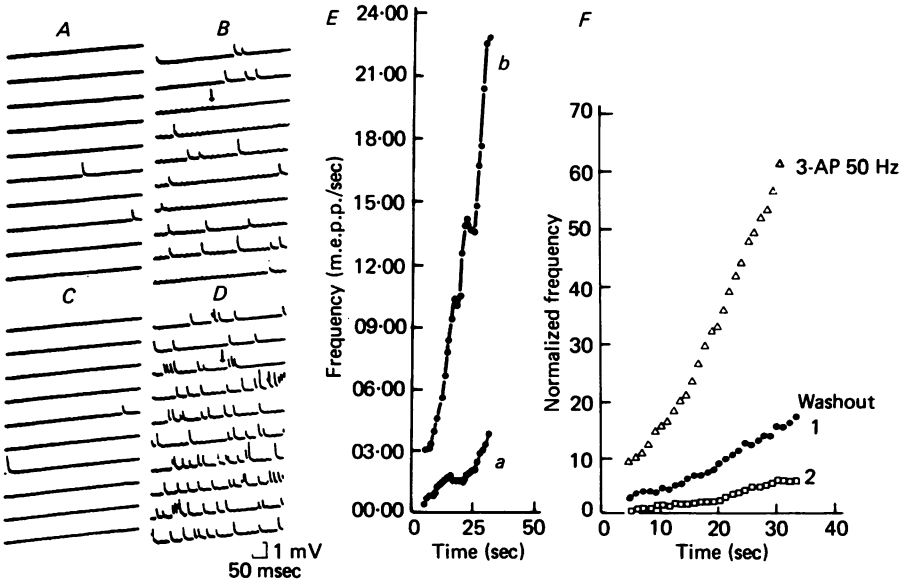

Fig. 6. The effects of the potassium channel blocker 3 aminopyridine (3-AP) on tetanic potentiation; inward electrochemical calcium gradient. *A* and *B*, sample records in control medium (50 μM-calcium chloride and 5·0 mM-magnesium chloride Ringer solution). *A* illustrates m.e.p.p. frequency before, and *B* during tetanic stimulation of 50 Hz for 40 sec. *C* and *D*, sample records in the same Ringer solution with 5 mM-3-AP. *C* illustrates m.e.p.p. frequency before, and *D* during tetanic stimulation of 50 Hz for 40 sec. *E*, tetanic potentiation (50 Hz for 40 sec) before (*a*) and after (*b*) the addition of 5 mM-3-AP. Moving bin, bin 10 sec Δ bin 1 sec. *F* from another preparation; tetanic potentiation in the presence of 3-AP (50 Hz for 40 sec) and during its washout, 20 (1) and 40 (2) min of washing with 50 μM-calcium chloride Ringer.

In post-tetanic potentiation, the most dramatic effect was the very rapid reduction in m.e.p.p. frequency immediately after the end of the stimulation period (Table 3). In control experiments the end tetanic frequency was $8{\cdot}74 \pm 2{\cdot}11$ and the frequency in the 5 sec immediately after the end of the tetanus was $7{\cdot}32 \pm 1{\cdot}58$ (a decrease to 83%). In the presence of 3-AP the end tetanic frequency was $77 \pm 13{\cdot}2$, and immediately after the end of stimulation it dropped to $27{\cdot}28 \pm 7{\cdot}02$ (a decrease to 35%). An examination of the time course of the post-tetanic decay showed that the augmentation phase was practically abolished already in nine out of eleven control experiments performed in a Ringer solution containing 5 mM-magnesium chloride; augmentation phase appeared in none of the experiments performed in the presence of the potassium channel blocker in spite of the huge increase in the end tetanic frequency (Fig. 7 and see Discussion). The potentiation phase persists with reduced time constant (Table 3) both under inward and reversed calcium electrochemical gradient. These results show that although tetanic and post-tetanic potentiation

TABLE 3. The effect of 3-aminopyridine on tetanic and post-tetanic potentiation at the frog neuromuscular junction

Composition of bathing medium	Frequency of stimulation (Hz)	Duration (sec)	Number of stimuli	Number of experiments	Excess quanta	$\frac{Q}{\text{impulse}}$	Normalized end tetanic frequency ± S.E. of mean	Normalized end tetanic frequency GA*	Normalized initial post-tetanic frequency ± SE of mean	Normalized initial post-tetanic frequency GA*	τ_p (sec)
Inward calcium gradient											
Control	50	40	2000	14	80·7 ± 20·6	0·0403	9·05 ± 1·65	5·86	7·74 ± 1·28	6·43	120·02 ± 16·67
5 mM-3-AP†	50	40	2000	5	595·9 ± 98	0·2979	77 ± 13·2	72·16	27·28 ± 7·02	23·42	75·62 ± 8·69
Reversed calcium gradient‡											
Control	100	40	4000	11	71·23 ± 31·82	0·0178	11·78 ± 1·99	11·84	24·3 ± 5·97	17·40	102·04 ± 10·78
5 mM-3-AP	100	40	4000	5	34·32 ± 4·91	0·0085	5·76 ± 1·74	4·34	7·36 ± 2·29	5·22	79·78 ± 7·36

* GA = geometric average.

† Inward calcium gradient: 0·05 mM-$CaCl_2$. 5 mM-$MgCl_2$ frog Ringer.

‡ Reversed calcium gradient: 2 mM-$MgCl_2$. 1 mM-EGTA frog Ringer.

are affected by a potassium channel blocker, potassium is not the main ion that induces potentiation in the absence of extracellular calcium.

Sodium and potentiation

The next step in the working hypothesis was to assume that the entry of sodium ions by the action potential induces the observed potentiation in the absence of an inward electrochemical gradient for calcium.

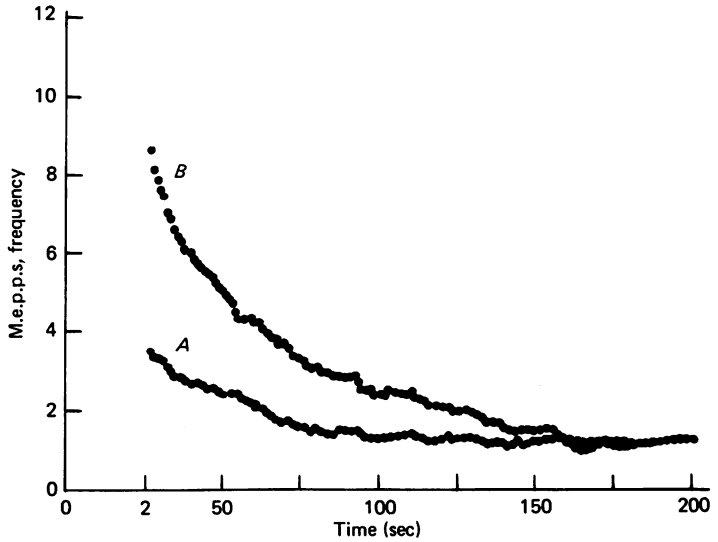

Fig. 7. The effect of the potassium channel blocker 3-aminopyridine (3-AP) on post-tetanic potentiation; inward electrochemical calcium gradient. The decay of post-tetanic potentiation following tetanic trains of 50 Hz for 40 sec, before (*A*) and after (*B*) addition of 5 mM-3-AP. Bin 50 sec, Δ bin 1 sec. 50 μM-calcium chrloride, 5 mM-magnesium chloride Ringer.

(1) *The effect of tetrodotoxin on potentiation*

If two ionic fluxes (sodium and calcium) participate in potentiation, then one can manipulate them separately. The inward calcium flux can be abolished by creating a reversed electrochemical gradient for calcium with EGTA, while the inward sodium flux can be inhibited by TTX. It has been shown before that TTX itself does not affect the basic properties of transmitter release (Katz & Miledi, 1967*a*).

Fig. 8 shows the effect of tetanic stimulation in the presence of TTX (1μg/ml.) in the perfusion medium. Fig. 8*A* illustrates an experiment where the electrotonic depolarization was performed in inward electrochemical gradient for calcium (200 μM). One can see that tetanic stimulation causes an increase in *f*, which decays slowly over a period of many seconds.

Afterwards, while the electrode was still in the same fibre, the external solution was changed to one containing no added calcium and 1mM-EGTA, to abolish the inward calcium concentration gradient across the membrane TTX (1 μg/ml. was

kept throughout the experiment). Now, stimulation of the motor nerve produces neither tetanic nor post-tetanic increase in f.

Similar observations were made at ten additional end plates.

These experiments show that it is enough to have either an inward calcium influx or an inward sodium influx to produce potentiation.

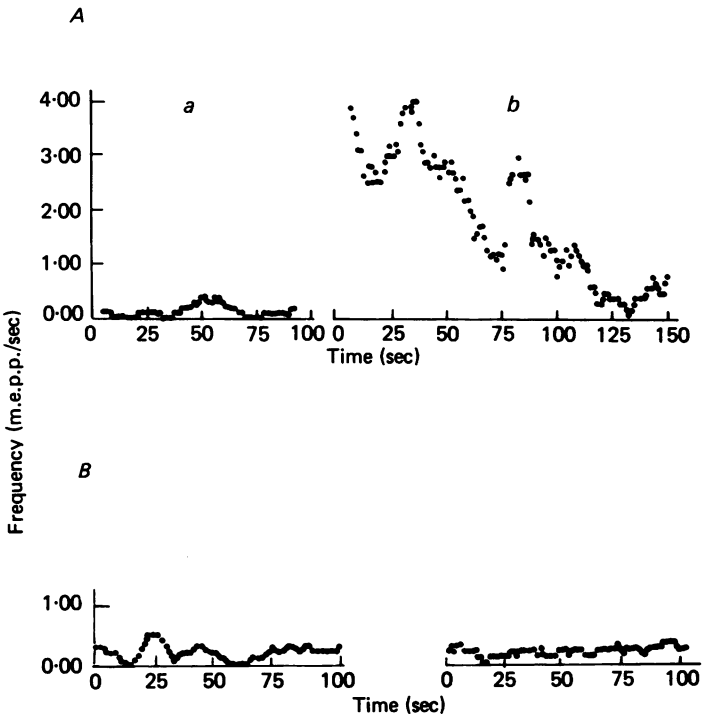

Fig. 8. The effect of sodium channel blockade on post-tetanic potentiation. *A*, inward electrochemical calcium gradient (200 μM-calcium chloride, 2·0 mM-magnesium chloride Ringer). *B*, reversed electrochemical calcium gradient (1 mM-EGTA, 2·0 mM-magnesium chloride Ringer) *a*, resting frequency; *b*, after a stimulation of 50 Hz for 40 sec; pulse duration 1 msec. 1 μg/ml. TTX, present through the experiment. Note that an inward calcium gradient is sufficient for generation of post-tetanic potentiation (*Ab*), but no post-tetanic potentiation is observed in *Bb*. Moving bin: bin 10 sec, Δ bin 1 sec.

(2) *Nerve terminal potential amplitude and tetanic potentiation*

When sodium is accumulated inside the nerve, the sodium gradient decreases and it is expected that the amplitude of the action potential will be reduced (Hodgkin & Katz, 1949). Unfortunately, the nerve terminal at the frog nerve muscle junction is too small to be penetrated safely with a micro-electrode. Hence, one has to settle on a less desirable procedure—monitoring the extracellular nerve terminal potential which is the time derivative of the intracellular potential change. Therefore one cannot distinguish between the reduction in action potential amplitude and slowing in the potential change.

Fig 9 shows that while the amplitude of the nerve terminal potential decreases, the frequency of the m.e.p.p.s increases, in a medium with a reversed electrochemical gradient for calcium.

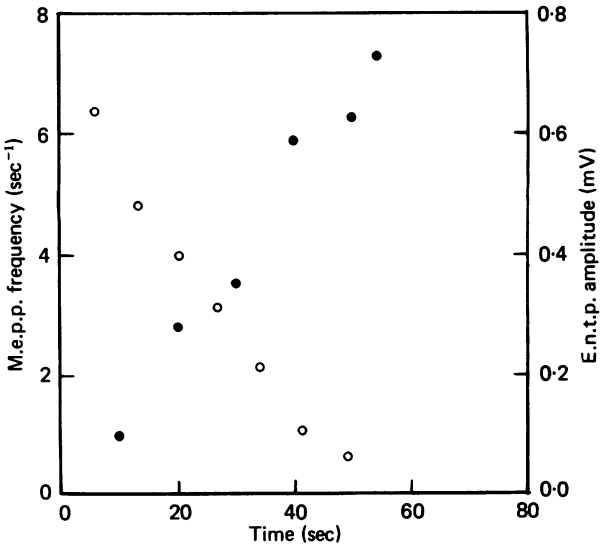

Fig. 9. An increase in miniature end-plate current frequency (filled circles) and a decrease in the averaged extracellular nerve terminal potential (open circles) during repetitive stimulation at 100 Hz for 60 sec. Focal extracellular recording by a calcium pipette, each point is the averaged response of 512 stimuli. Reversed calcium gradient conditions 2 mM-magnesium chloride, 1 mM-EGTA Ringer with no added calcium.

(3) *The effect of ouabain on the time course of potentiation*

It is well known that addition of a sodium pump inhibitor such as ouabain causes, after a delay of 10–20 min, an increase in spontaneous and evoked transmitter release (Birks & Cohen, 1968*a*, *b*). This facilitatory effect of ouabain on transmitter release does not require the presence of extracellular calcium (Baker & Crawford, 1975).

We examined the effect of ouabain on the time course of post-tetanic potentiation in the absence of extracellular calcium. It is expected that if accumulation of sodium is a contributor to tetanic and post-tetanic potentiation, then inhibition of the Na-K ATPase and the extrusion of sodium will slow the decay of post-tetanic potentiation. Fig. 10 shows that this is indeed the case: Addition of 5×10^{-5} g/ml. ouabain increased dramatically τ_p (the mean increase was from 102·04 to 215 sec).

The lack of preferable timing of sodium induced transmitter release

There are two alternatives for the action of sodium on transmitter release: they can either cause directly the exocytotic process or they may have an indirect action, such as releasing calcium from intracellular stores. If the action of sodium is direct on the release process, it is expected that the maximal action will be shortly after the action potential, when the sodium ion concentration will be maximal at the

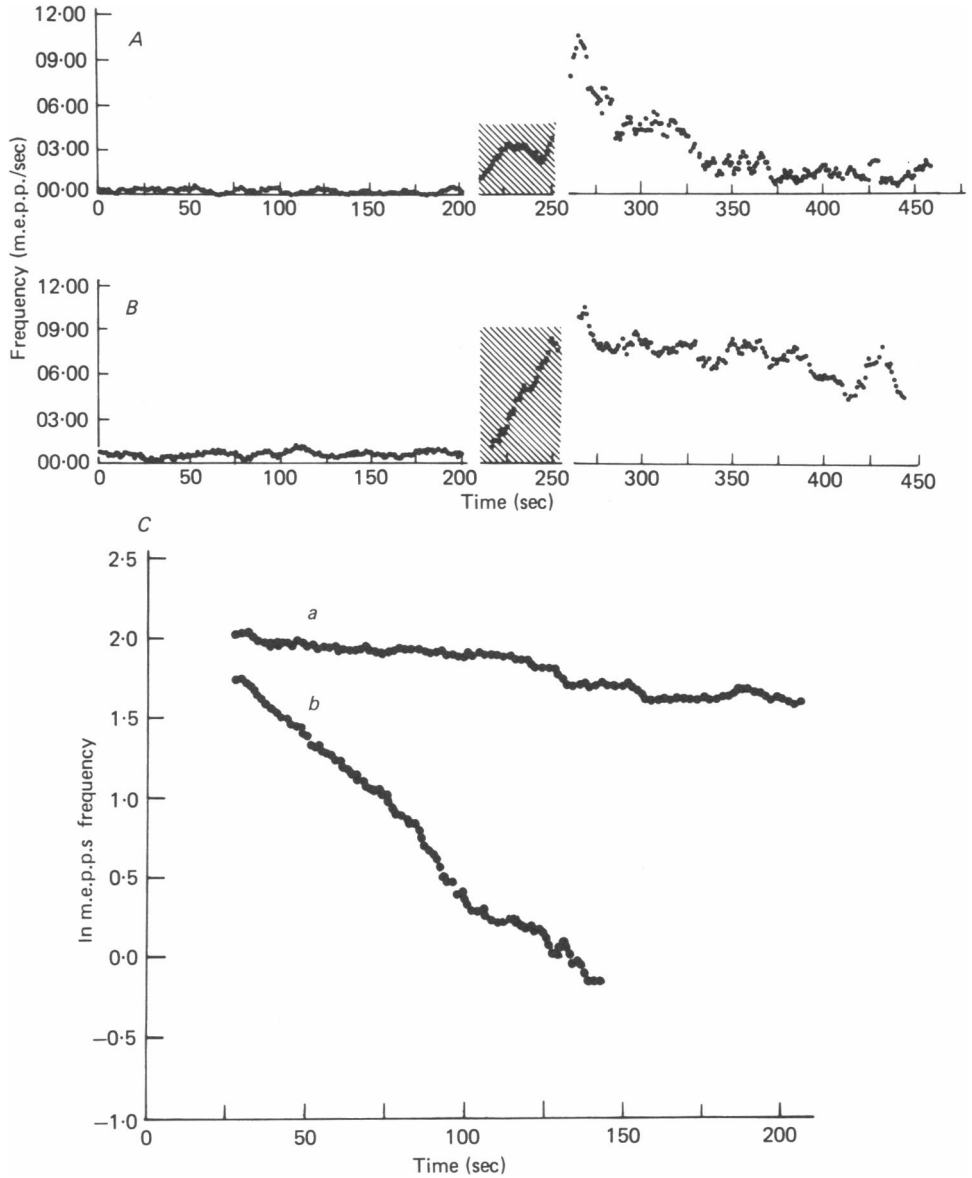

Fig. 10. Effect of ouabain on post-tetanic potentiation. The time courses shown in *A* and *B* represent the resting frequency (left), the tetanic increase (dashed) and the post-tetanic decay (right) of m.e.p.ps frequency. *A* before, *B* after the addition of ouabain 5×10^{-5} g/ml. Tetanic trains at 100 Hz for 50 sec were given to the nerve under reversed calcium gradient conditions (2 mM-magnesium chloride, 1 mM-EGTA Ringer with no added calcium). Moving bin: bin 10 sec, Δ bin 1 sec. *C* shows post-tetanic decays (*A*) before and (*B*) after addition of ouabain to the medium, on a semilogarithmic plot, from another end-plate. Moving bin: bin 50 sec, Δ bin 1 sec.

critical sites for release. To examine this question, we measured the time of appearance of the m.e.p.p.s after tetanic nerve stimulation. Fig. 11*A* shows that in the absence of extracellular calcium, there is an increase in the frequency of m.e.p.p.s, but there is no significant preferable timing for this action; the increase in f is

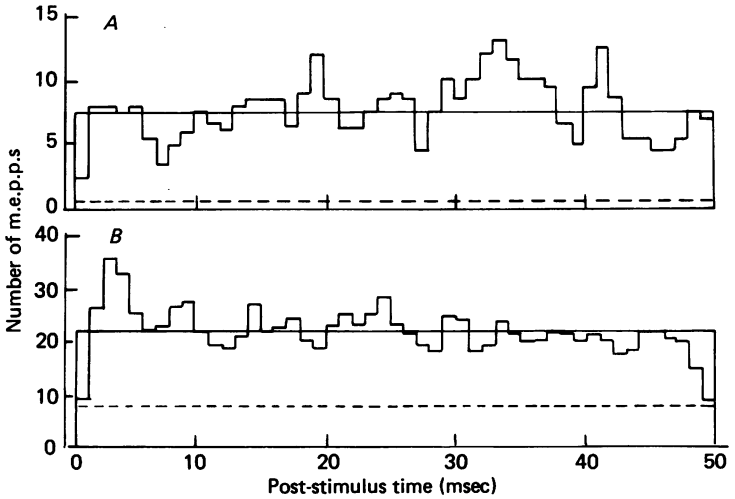

Fig. 11. The time of appearance of miniature end-plate potentials during tetanic stimulations. *A*, post stimulus histograms of m.e.p.p.s during a tetanic stimulation of 16 Hz for 297 sec in reversed calcium gradient (2 mM-magnesium chloride, 1 mM-EGTA frog Ringer with no added calcium); it shows no preferred time of appearance of m.e.p.p.s, whereas in *B* a typical time locked appearance is observed during a tetanic stimulation at 16 Hz for 80 sec in inward calcium gradient conditions (50 μM-calcium chloride, 2 mM-magnesium chloride Ringer). The horizontal lines represent the mean expected number of m.e.p.p.s. The dashed lines show the resting, prestimulation number of expected m.e.p.p.s. Moving bin: bin 2 msec, Δ bin 1 msec.

unrelated to the timing of the action potential. It is enough to have only a very small amount of calcium ions in the extracellular medium (50 μM) to produce a clear peak shortly after nerve activation (Fig. 11*B*).

The effects of magnesium on tetanic and post-tetanic potentiation

Magnesium ions compete with calcium in the entry process through the nerve membrane (Baker, 1976; Rojas & Taylor, 1975). If calcium influx is one of the processes involved in tetanic potentiation, then it is expected that an increase in $[Mg]_o$, under conditions of an inward electrochemical gradient for calcium ions, will cause a decrease in tetanic potentiation. This expectation was indeed fulfilled: in eleven experiments performed in 50 μM calcium chloride and 2·0 mM-magnesium chloride, the relative normalized frequency of the m.e.p.p.s near the end of a tetanus of 50 Hz for 40 sec, was $17{\cdot}23 \pm 7{\cdot}1$. At the same end-plates an increase in $[Mg]_o$ to 5·0 mM caused a decrease in end tetanic frequency to $8{\cdot}47 \pm 2{\cdot}11$. An example of this effect is illustrated in Fig. 12*A*. A further increase of $[Mg]_o$ to 10 mM produced variable results according to the length of exposure of the preparation to high magnesium; the initial effect was a further decrease in the end tetanic frequency;

but if the preparation was bathed for hours in high magnesium a progressive increase in the m.e.p.p. frequency was observed.

If magnesium ions inhibit not only the entry of calcium, but also the efflux, then according to this hypothesis two effects of magnesium can be predicted. First, an increase in $[Mg]_o$ will reduce the efflux of calcium during the tetanic stimulation under reversed calcium gradient, hence the tetanic potentiation will increase in

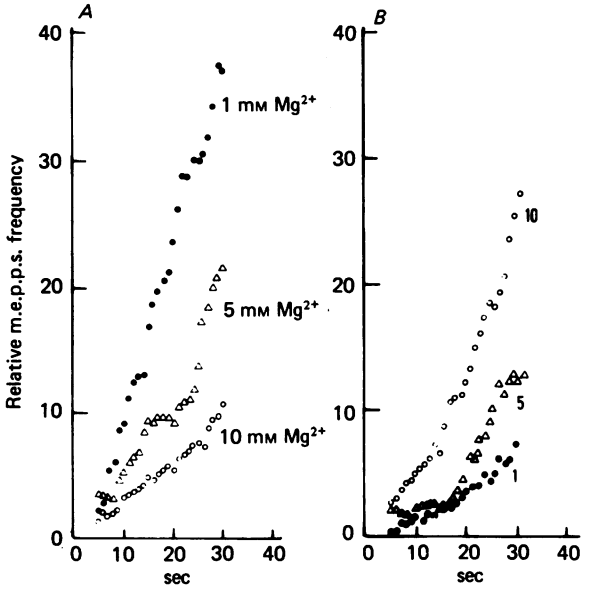

Fig. 12. The effect of magnesium on tetanic potentiation depends on the direction of the calcium electrochemical gradient. *A*, inward electrochemical gradient; elevation in $[Mg]_o$ (concentrations marked near each set of points) causes a *decrease* in tetanic potentiation (50 Hz for 40 sec). 50 μM-calcium chloride Ringer solution. *B*, reversed electrochemical gradient; elevation in $[Mg]_o$ (concentration marked near each set of points) causes an increase in tetanic potentiation (100 Hz for 40 sec). 1 mM-EGTA with no added calcium Ringer solution. Moving bin: bin size = 10 sec, Δ bin 1 sec.

$[Mg]_o$. In six experiments the end tetanic frequency and the number of quanta released by an impulse increased indeed upon elevation of $[Mg]_o$. Tetani of 100 Hz for 40 sec produced end tetanic frequencies of $4{\cdot}62 \pm 0{\cdot}56$, $16{\cdot}5 \pm 1{\cdot}06$ and $19{\cdot}4 \pm 4{\cdot}41$ in 2·0, 5·0 and 10 mM $[Mg]_o$ respectively; the number of excess quanta/impulse were $0{\cdot}0053 \pm 0{\cdot}0008$, $0{\cdot}0162 \pm 0{\cdot}002$, $0{\cdot}0223 \pm 0{\cdot}005$ in 2·0, 5·0 and 10 mM $[Mg]_o$. Fig. 12*B* illustrates the effects of magnesium ions under reversed electrochemical gradient for calcium. These results can be explained of course in an alternative way; namely, that the action potentials increase magnesium conductance, magnesium ions flow inside the nerve terminal and activate *directly* the quantal release mechanism. Such an explanation does not seem very likely in view of the lack of preferred time intervals after the nerve stimulus (Fig. 13).

The second prediction deals with the post-tetanic jump; if calcium conductance is decreased by magnesium, then the outward leakage of calcium from the terminal will be smaller during the tetanus and the post-tetanic jump will, therefore, be

smaller. In six experiments (at six different end-plates) performed in 2·0 mM-magnesium chloride in the outside medium, the ratio f_{ps} (the post-tetanic frequency during the first 5 sec after the tetanus) $/f_s$ (the end tetanic frequency) was $1{\cdot}85 \pm 0{\cdot}36$, while in nine experiments performed at the same end plates in 5·0 or 10·0mM-magnesium chloride the ratio f_{ps}/f_s was $1{\cdot}03 \pm 0{\cdot}07$.

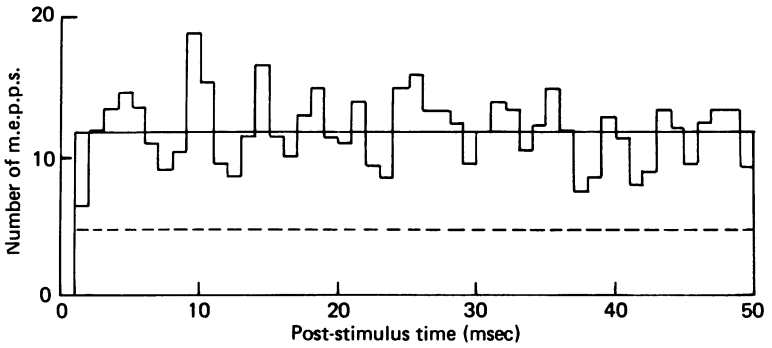

Fig. 13. The time of appearance of miniature end-plate potentials after nerve stimulation (16 Hz for 99 sec). No preferred time of appearance. The horizontal line represents the mean expected number of m.e.p.p.s, the dashed line represents the expected number of m.e.p.p.s if the resting frequency had continued during the stimulation period. 5 mM-magnesium chloride, 1 mM-EGTA Ringer with no added calcium. Moving bin: bin = 2 msec, Δ bin 1 msec.

The rate of decay of potentiation was slower at higher concentrations of magnesium chloride. τ_p was increased from $80{\cdot}5 \pm 8{\cdot}65$ S.E. of mean ($n = 24$) in 50 μM-calcium chloride, 2 mM-magnesium chloride Ringer, to $144{\cdot}09 \pm 20$ ($n = 19$) in 50 μM-calcium chloride, 5 or 10 mM-magnesium chloride Ringer, in response to stimulation of 50 Hz for 40 sec.

In reversed calcium gradient, following tetani of 100 Hz for 40 sec, τ_p in 2 mM-magnesium chloride frog Ringer was $102{\cdot}04 \pm 10{\cdot}78$ S.E. of mean ($n = 11$) and was prolonged to $129{\cdot}26 \pm 14{\cdot}7$ S.E. of mean ($n = 9$) in frog Ringer which contained 5 or 10 mM-$MgCl_2$.

DISCUSSION

Processes participating in tetanic and post-tetanic potentiation

In the present work the increase in the rate of release of acetylcholine quanta by repetitive nerve stimulation was taken as an indication of tetanic and post-tetanic potentiation. It can be inferred that at least three separate processes seem to take part in tetanic potentiation: influx of calcium, intracellular calcium translocation and changes in the amplitude of the presynaptic action potential. Two of them augment transmitter release, while one presumably suppresses it. The first is the well known increase in the calcium permeability by depolarization (Katz & Miledi, 1967*a*, *b*, 1969; Baker, Hodgkin & Ridgway, 1971; Llinas & Nicholson, 1976). This increase in G_{Ca} causes a large increase in the number of quanta liberated by the nerve impulse. Factors that increase further this G_{Ca} (such as 3-aminopyridine

that prolongs the action potential and thus increases the voltage and the time dependent calcium permeability) cause an increase in tetanic potentiation, while factors that inhibit the calcium influx (such as $[Mg]_o$), or reduce calcium influx (reversed electrochemical gradient for calcium) suppress tetanic potentiation.

The decrease in the extracellularly recorded averaged action potential (Fig. 9) was taken to indicate a reduction in action potential amplitude. This is not an accurate measure of the intracellular action potential amplitude; being a time derivative of the intracellular changes it also may reflect a slowing of the action potential configuration. But the small dimensions of the nerve terminal at the frog neuromuscular junction do not allow better measurements at present. It has been shown previously (Katz & Miledi, 1967*a*, *b*) that a decrease in presynaptic depolarization reduces the amount of transmitter liberated, probably by preventing the calcium channels to fully open. Such a dependence of calcium conductance on the degree of membrane depolarization has been described also in a number of other systems (Beeler & Reuter, 1970; Mironneau, 1973; Baker, Meves & Ridgway, 1973; Hagiwara, Ozawa & Sand, 1975). This decrease in the amplitude of the presynaptic action potential develops gradually during the high frequency stimulation of the motor nerve until eventually a failure in the propagation of the action potential occurs (Krnjevic & Miledi, 1959).

In spite of the decrease in the action potential amplitude and even in solutions generating reversed electrochemical gradient for calcium across the presynaptic membrane, the frequency of the m.e.p.p.s increases upon tetanic nerve stimulation. The proposed explanation for this component of tetanic potentiation is that the nerve stimulation causes a translocation of calcium ions from intracellular stores into the cytosol near the active zones for release. Three types of questions arise regarding this calcium translocation. How is the coupling between nerve terminal activity and translocation achieved? What are the subcellular sources for this calcium translocation? And finally, is this calcium translocation of importance under normal physiological conditions?

Coupling

The present work indicates that sodium ions can serve as a coupling element between nerve terminal repetitive activity and increase in transmitter release. Three lines of evidence are consistent with this notion: the lack of potentiation when the nerve terminal is depolarized in the presence of the sodium channel blocker, TTX (Fig. 8), the facilitating effect of the sodium pump inhibitor, ouabain, on potentiation (Fig. 10) and the decrease of the extracellular action potential during tetanic stimulation (Fig. 9). The lack of a preferred time interval for the release of acetylcholine quanta suggests that the effect of sodium is presumably not by a direct activation of the transmitter release action (Fig. 11).

The notion that intracellular sodium ions can induce transmitter release is not new. It has been suggested by Birks & Cohen (1968*b*) and by Baker & Crawford (1975) for vertebrate preparation and by Atwood, Swenarchuk & Gruenwald (1975) and Swenarchuk & Atwood (1975) for invertebrate preparations.

Sodium ions need not be the only coupling factor between the nerve terminal

activity and augmentation of transmitter release during tetanic stimulation. The secondary effect of magnesium on transmitter release may also be an indication of a possible coupling role.

Source of translocated calcium

Only a small fraction of the total intracellular calcium is in a free ionized form (see Baker, 1976; Rahamimoff, 1979). Most of it is bound by subcellular organelles and buffers. They include mitochondria (see Alnaes & Rahamimoff, 1975) vesicles (Politoff, Rose & Pappas, 1974; Rahamimoff & Abramovitz, 1978*a*, *b*), endoplasmic reticulum (Henkart, Reese & Brinley, 1978), membranes (Baker & McNaughton, 1978) and soluble buffers (see Baker & Schlaepfer, 1978).

Potentially every one of these components can contribute the necessary calcium for translocation. It is of interest to note that Carafoli & Crompton (1978) have shown recently that relatively small changes in sodium concentrations are sufficient to produce liberation of calcium from brain mitochondria.

The effect of sodium on calcium metabolism need not be a direct one. The nerve terminal contains extra-mitochondrial ATP-dependent calcium uptake systems (Blaustein *et al.* 1978). Recently the importance of direct involvement of ATP in calcium extrusion has been stressed (Dipolo, 1978; Dipolo & Beauge, 1979). Therefore, it is feasible that extensive usage of ATP by the sodium extrusion mechanism decreases the availability of ATP for the ATP-dependent calcium efflux thus increasing free $[Ca]_{in}$ and transmitter release.

Is the calcium translocation process of physiological importance?

The involvement of an intracellular calcium translocation in tetanic potentiation has been shown in this work under conditions of low extracellular $[Ca]_o$ and thus low quantal content. Although the relative potentiation contributed by this factor is quite large, its absolute magnitude is rather small under the specific experimental conditions employed here. If the relation between free $[Ca]_{in}$ and transmitter release is sigmoidal (similar to the relation between $[Ca]_o$ and release, Jenkinson, 1957; Dodge & Rahamimoff, 1967; Hubbard, Jones & Landau, 1968; Katz & Miledi, 1969), then even small changes in $[Ca]_{in}$ can have considerable effect on physiological transmitter release; if, on the other hand, release of transmitter is a linear function of $[Ca]_{in}$, as suggested by Llinas, Steinberg & Walton (1976), then the importance of this factor will be minimal. At present, however, there are only indirect indications that the relation between $[Ca]_{in}$ and release is more than linear (see Rahamimoff, Lev-Tov, Meiri, Rahamimoff & Nussinovitch, 1979). Therefore, the assessment of the physiological significance of this proposed calcium translocation has to await the resolution of the issue regarding the $[Ca]_{in}$-transmitter release relation.

Post-tetanic potentiation

Magleby & Zengel (1975, 1976) have described three distinct phases in post-tetanic potentiation of evoked transmitter release: facilitation, augmentation and potentiation. The first one is very short (about 1 sec) and is outside the resolution of our experimental procedures employing miniature end plate potentials as an indication for the probability of transmitter release. The other two phases are clearly seen

when the nerve terminal is intensively stimulated in the presence of calcium ions in the extracellular medium (Table 1). The faster augmentation phase did not appear usually under slow rates of stimulation, reversed electrochemical gradient for calcium and when the extracellular medium contained magnesium ions above 5 mM. There are at least two possible explanations for the augmentation phase: either it represents a residual calcium conductance that persists for seconds after the end of the tetanic stimulation or it is a consequence of the higher frequency at the end of the tetanus and reflects presumably the processes responsible for reducing the intracellular calcium towards its resting value. Of these two hypotheses we consider the former to be of a greater importance since it is possible to induce very high end tetanic m.e.p.p. frequencies without generating augmentation. One such example is the action of the potassium channel blocker, 3-AP, which causes very large increases in the end tetanic frequency without inducing augmentation.

Since the influence of the proposed calcium translocatory process increases with the duration and the frequency of stimulation and so does the time constant of the decay of potentiation, we suggest that it constitutes a major component of potentiation. This suggestion is strengthened by the prolongation of the potentiation by ouabain. If this hypothesis is correct, then the activity of the surface-membrane sodium pump, mitochondrial function and the metabolic energy of the nerve terminal become important determinants in the regulation of synaptic transfer efficiency.

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