J. Physiol. (1969), **203**, *pp.* 121–133
With 6 text-figures
Printed in Great Britain

POST-TETANIC POTENTIATION AT THE NEUROMUSCULAR JUNCTION OF THE FROG

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(*Received* 30 *December* 1968)

SUMMARY

1. Post-tetanic potentiation (PTP) was studied at the neuromuscular junction of the frog. The magnitude and time course of PTP was dependent on the number of stimuli in the tetanus, rather than on the frequency or duration of the tetanus. At low temperature the maximum amplitude of PTP was unchanged, but the time course was prolonged.

2. The magnitude and time course of PTP was accounted for quantitatively by estimated changes in the fraction of transmitter released per stimulus.

3. As external [Ca] was reduced, the time for decay of PTP was decreased. The maximum amplitude of PTP, measured with respect to control amplitude at the same [Ca], was unchanged, but occurred at earlier times. The time course of PTP was dependent only on the [Ca] present during the tetanus.

4. It is concluded that PTP is associated with an intracellular accumulation of Ca during the tetanus.

INTRODUCTION

Following a train of stimuli to the presynaptic nerve, the end-plate potential (e.p.p.) produced by a test stimulus is increased in amplitude relative to control responses. This period of post-tetanic potentiation may last from several seconds to several tens of minutes depending on the parameters of the tetanic stimulation (Feng, 1941; Larrabee & Bronk, 1947; Liley & North, 1953; Gage & Hubbard, 1966; Braun, Schmidt & Zimmerman, 1966). The potentiation is due to increased release of transmitter from presynaptic endings (Hutter, 1952; Liley, 1956) and may be distinguished from the early brief phase of facilitation observable after a single stimulus (Hubbard, 1963). In the first part of the present study the dependence of PTP on stimulus parameters has been reinvestigated. It was found that the time course of PTP is dependent on the number of

stimuli in the tetanus, rather than the frequency or duration of the tetanus. Evidence was obtained which suggests that PTP is a result of an increased fraction of the transmitter being released by an action potential, rather than an increase in the amount of the transmitter available for release.

It was also of interest to investigate the role of Ca in the production of PTP. Transmitter release depends on the presence of Ca (e.g. Katz & Miledi, 1965), and the present study demonstrates that PTP also depends on the external Ca concentration at the time the tetanus is given.

METHODS

The frog sartorius nerve–muscle preparation was used in all experiments. In many experiments the muscle was suspended in a moist air chamber and extracellular recordings of the e.p.p.s were obtained by recording the potential difference between two platinum wire electrodes placed across the end-plate region and the pelvic end of the muscle. In other experiments e.p.p.s were recorded intracellularly using KCl-filled micropipettes. In all cases supramaximal nerve stimulation was used. Tetanic stimulation ranged from frequencies of 35–150/sec, with train durations of 0·5–60 sec. In most experiments test stimuli were delivered at 30 sec intervals during the post-tetanic period.

The normal saline solution consisted of (mM):NaCl 115, KCl 2·0, $CaCl_2$ 1·8, $NaHCO_3$ 2·4. Neuromuscular transmission was blocked by adding (+)-tubocurarine chloride (TC) to the bathing solution in concentrations of 0·5–10 μg/ml., or by adding appropriate amounts of $MgCl_2$. When $MgCl_2$ was added, it replaced an osmotically equivalent amount of NaCl. Changes in other ions were made by simple addition or deletion of the ion from the solution, since the resulting deviations from isosmolarity were small. In some intracellular experiments the bathing fluid was changed during the course of an experiment to achieve a different fluid composition during the tetanic stimulation. In such experiments the e.p.p. amplitudes during control washes indicated the time course of the wash in and wash out. In some experiments a Peltier cell was used to cool the bath and preparation, whose temperature was measured with a bead thermistor near the region of recording. In such extracellular experiments the whole muscle was immersed in oil, rather than in moist air, to ensure stable temperature conditions.

RESULTS

Post-tetanic potentiation in curarized preparation

After tetanic stimulation of the motor nerve there was an initial period during which the amplitude of a single test e.p.p. was depressed, compared with the control e.p.p., followed by a period during which the e.p.p. amplitude was potentiated. At maximum potentiation the amplitude was usually between 140 and 220 % of its control value. The time to peak and the time course of decay depended on the frequency and duration of the stimulus train. In TC at least 800 stimuli were required before PTP became apparent. Figure 1 shows the effect of frequency on PTP. The train duration was fixed at 20 sec. As the frequency of stimulation was increased, up to about 1300 stimuli (62·5/sec for 20 sec) both the amplitude and the time course of PTP increased. When the number of stimuli exceeded 1300, there

was a further increase in the time course alone, seen as both an increase in time to maximum amplitude and a prolonged decay (cf. Curtis & Eccles, 1960). In an analogous manner, an increase in the duration of stimulation at a fixed frequency caused a marked increase in the time course of PTP (cf. Feng, 1941; Larrabee & Bronk, 1947; Liley & North, 1953).

Further experiments demonstrated that the amplitude and time course of PTP were directly related to the number of stimuli delivered, rather than to frequency or duration alone. In Fig. 2*A* it can be seen that a train

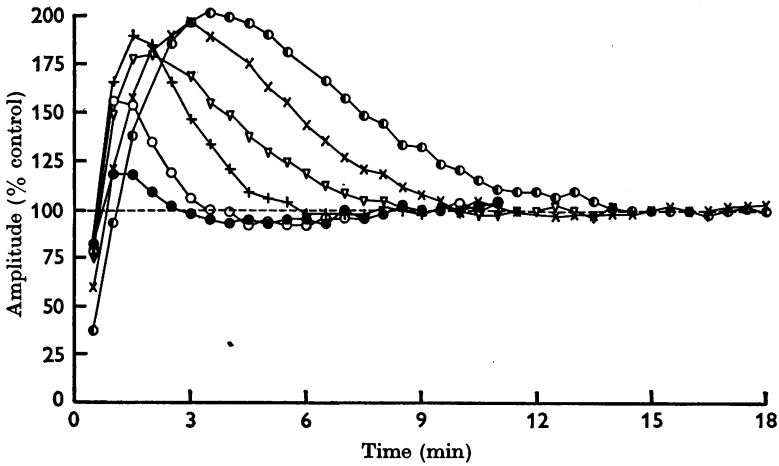

Fig. 1. Variation of e.p.p. amplitude following tetani of different frequency. Recordings were obtained extracellularly from the end-plate region. TC 10 μg/ml. Trains were 20 sec in duration. Train frequencies were (in order given): + 62·5/sec; ● 40/sec; × 100/sec; ○ 50/sec; ◐ 125/sec; ▽ 77/sec. The first test stimulus was given 10 sec after the end of the train, and subsequent tests were given at 30 sec intervals. In this and similar figures the ordinate is the amplitude of the e.p.p. as a percentage of the control amplitude, and the abscissa is the time after the beginning of the tetanus.

at 50/sec for 20 sec produced virtually the same potentiation as a train at 100/sec for 10 sec. This raised the question whether PTP is related to the amount of transmitter released during the train. That this is not the case can be seen in Fig. 2*B*, where the amplitudes of successive e.p.p.s *during* the trains are plotted. The e.p.p. amplitudes, and therefore the amount of transmitter released by each shock, are consistently larger during the lower frequency tetanus, the summed amplitudes being approximately 1·7 times greater than during the higher frequency train. The steady-state e.p.p. amplitude at the end of the lower frequency train is 2·3 times greater. This indicates that neither the amount of transmitter released during the train nor the final level of release at the end of the train determines the magnitude and the time course of PTP. The major determining factor, then,

seems to be the number of stimuli applied to the nerve, suggesting that PTP may be a result of some change associated with nerve activity rather than transmitter release.

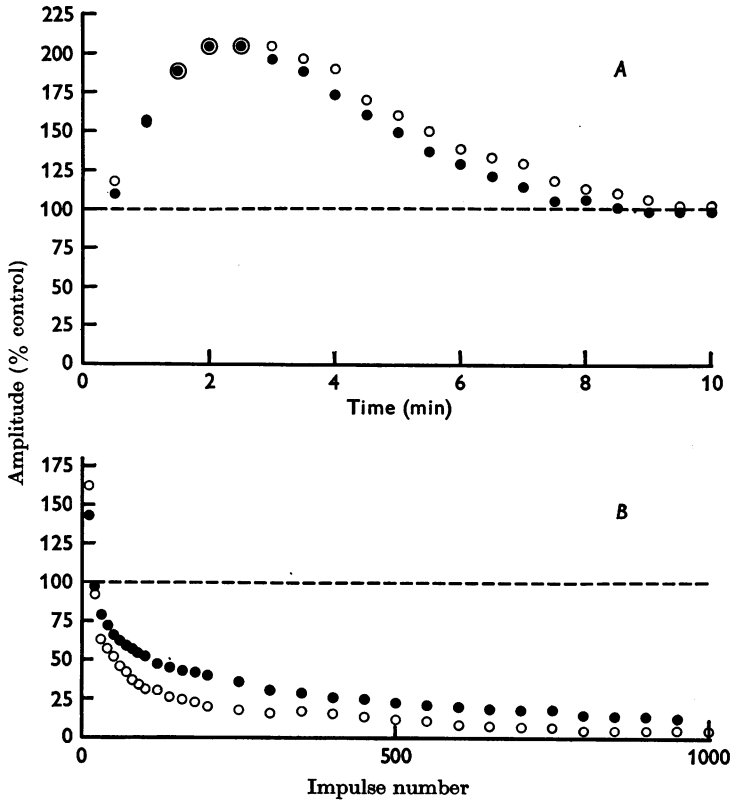

Fig. 2. *A*. Variation of e.p.p. amplitude following tetani with fixed numbers of stimuli. Recordings were obtained extracellularly from the end-plate region. TC 10 μg/ml. The trains were 50/sec for 20 sec (●) and 100/sec for 10 sec (○).

B. Relative amplitude of e.p.p.s during tetanic stimulation. The same experiment as in *A*. Each point represents an average of 10 successive amplitudes. From 1 to 100 stimuli, successive groups of 10 stimuli were averaged; from 100 to 200 stimuli, every second group of 10 stimuli were averaged; from 200 to 1000 stimuli, every fifth group of 10 stimuli were averaged. The area under the curves is proportional to the amount of transmitter released during the trains and is 1·7 times greater at 50/sec for 20 sec than at 100/sec for 10 sec.

Several experiments were performed to test the effects of temperature on PTP. In all cases the maximum amplitudes of PTP at 7–12° C was the same as at room temperature (~ 20° C); but, as can be seen in Fig. 3*A*, the time course was greatly prolonged. For a decrease of about 8·5° C, the time for decay of PTP to half-amplitude increased about 2·5 times. Such

a large temperature effect is consistent with a complex process which may or may not be metabolically dependent. An additional finding was that the onset of PTP appeared to be separated from the recovery from post-tetanic depression by an inflexion. This can be seen in Fig. 3*B*, in which the averaged results obtained from three experiments at 11·5° C during the first 5 min after the tetanus are plotted on an expanded time scale. During this period the amplitudes of the test e.p.p.s were initially depressed and recovered toward the control value with an apparent exponential time

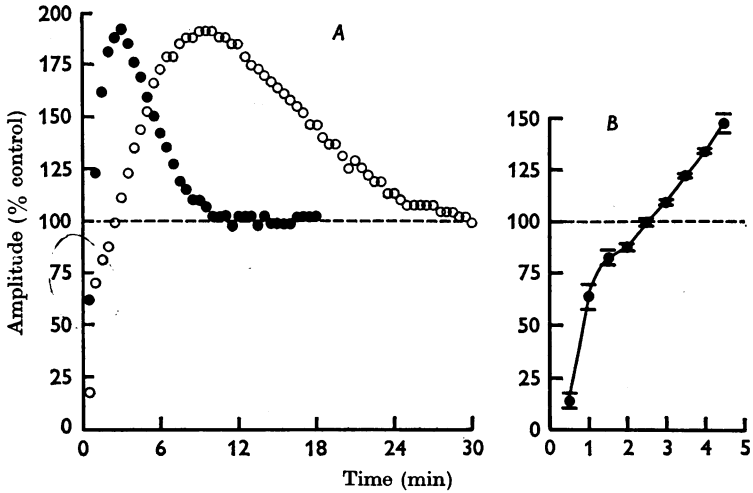

Fig. 3. *A*. Effect of temperature on PTP. Recordings were obtained extracellularly from the end-plate region. TC 10 μg/ml. Tetani were 62·5/sec for 20 sec. The temperature was 20·2° C (●) and 11·5° C (○).

B. Changes in the e.p.p. amplitude during the early post-tetanic period in the cold. The values are the average of three experiments and the bars represent ± 1 S.D. TC 10 μg/ml. The temperature was 11·5° C and tetani were 50/sec for 20 sec.

course having a time constant of about 1 min. About 1–2 min after the end of the tetanus the rate of increase of e.p.p. amplitude with time became more rapid, presumably indicating the onset of PTP. This finding suggests that a depression was not masking a large 'zero time' potentiation, but that the potentiation did not begin until after the termination of the tetanus. Further evidence for this is given below.

It would be of interest in the framework of the quantal hypothesis to relate PTP to changes in the number of quanta of ACh available for release (n) or to the probability of release of the quanta (p) (del Castillo & Katz, 1954). One method of estimating p is to give two stimuli in an interval beyond the period of facilitation but within the period of depression. The fundamental assumption is that the depression of the second response is due solely to depletion of the store of available quanta. With this assump-

tion, an increase in p during the post-tetanic period will lead to a corresponding increase in depression of the second response, expressed as a fraction of the amplitude of the first response. An increase in n, on the

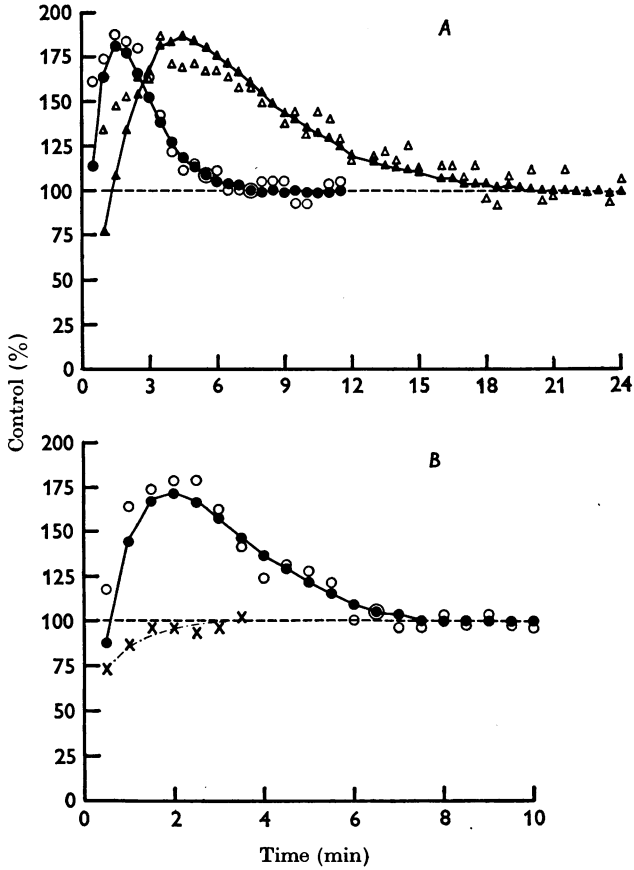

Fig. 4. *A*. Changes in e.p.p. amplitude (filled symbols) and estimated changes in p (open symbols) during the post-tetanic period. Recordings were obtained extracellularly from the end-plate region. TC 10 μg/ml. The test sequence consisted of two stimuli at an 800 msec interval, delivered every 30 sec. See text for method of estimating p. Tetani were 50/sec for 20 sec (● ○) and 100/sec for 20 sec (▲ △).

B. Changes in e.p.p. amplitude (●) and estimated changes in p (○) and n (×) during the post-tetanic period. The points are the averaged values from nine experiments. Recordings were obtained extracellularly from the end-plate region. TC 10 μg/ml. Tetani were 50/sec for 20 sec.

other hand, will produce no such change in the relative amount of depression. If n_0 is the amount of transmitter initially available, and p is the release probability, the first stimulus will release $m_1 = n_0 p$ quanta, and there is immediately available for release by a second stimulus $n_0 - m_1$

quanta. If the deficit is replenished exponentially with a time constant τ (Takeuchi, 1958; Thies, 1965), a second stimulus at a time Δt after the first will release

$$m_2 = m_1\{1 - p\exp(-\Delta t/\tau)\}.$$

Then

$$p = (1 - m_2/m_1)\exp(\Delta t/\tau).$$

If τ is a constant, the ratio of the release probability in the post-tetanic period (p_t) to that in the control period (p_c) will be given by

$$p_t/p_c = (1 - m_2/m_1)_t/(1 - m_2/m_1)_c.$$

Figure 4*A* shows the results of such measurements following two different frequencies (circles and triangles). The test stimuli were 800 msec apart and were delivered every 30 sec during the post-tetanic period. The filled symbols represent the amplitude of the first response in each test sequence relative to the amplitude of the first response in identical control sequences, i.e. a plot of PTP as in the preceding Figs. The open symbols represent the ratio p_t/p_c. The estimated changes in p coincide closely with the magnitude and time course of PTP, except during the rising phase. These results confirm quantitatively the earlier observations that PTP is due to an increase in release probability rather than an increase in available transmitter (Liley & North, 1953). Of particular interest was the observation that the probability estimates made immediately after the train were near the control value, again indicating that the early depression was not masking any large 'zero time' potentiation. The early depression may be attributed to a decrease in n as a result of the transmitter depletion during the train (Liley & North, 1953; Thies, 1965). In Fig. 4*B*, results from nine experiments have been averaged. The crosses represent the estimated relative values of n in the post-tetanic period (n_t/n_c) and show recovery from the apparent depression. Estimates of n_t/n_c were made by dividing the relative e.p.p. amplitude by the relative value of p. The time constant of recovery from post-tetanic depression, about 45 sec, is more prolonged than the time constant of 5–7 sec seen after one or a few stimuli (Takeuchi, 1958).

The effect of calcium

The fact that PTP appears to be dependent on the number of stimuli given to the nerve suggests that it may be a result, directly or indirectly, of one or more of the ionic changes associated with an action potential at the nerve terminal. Experiments were therefore designed to measure the effect of external [Ca] on PTP. Figure 5*A* shows the effects of varying external [Ca] in the range from 0·15 to 1·8 mM. The maximum PTP, measured as the ratio of the post-tetanic e.p.p. amplitude to control amplitude in the same solution, is independent of the [Ca]. This means that

in low [Ca] the *absolute* post-tetanic increase in e.p.p. amplitude was proportionately less than in solutions with higher control levels of release. The most pronounced effect of low [Ca] was on the time course of PTP. As Ca was reduced the time for decay to half-amplitude was decreased (Fig. 5*B*). It can also be seen that as [Ca] was decreased the post-tetanic depression decreased, presumably because of reduced depletion of transmitter during the tetanus. The time to maximum PTP was also decreased

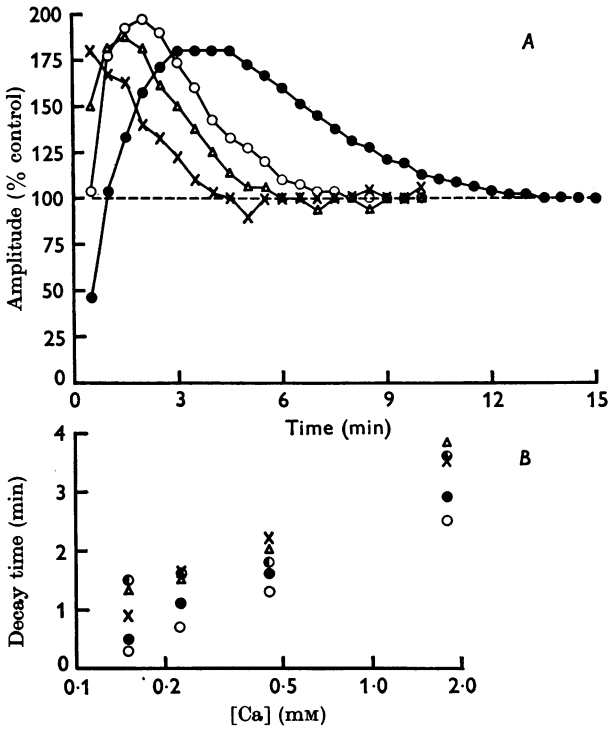

Fig. 5. *A*. Effect of external [Ca] on PTP. Recordings were obtained extracellularly from the end-plate region. Normal [Ca] is 1·8 mM. ● 1 × Ca (TC 10 μg/ml.); ○ $\frac{1}{4}$ × Ca (TC 3 μg/ml.); △ $\frac{1}{8}$ × Ca (TC 1 μg/ml.); × $\frac{1}{12}$ × Ca (TC 0·5 μg/ml.). The e.p.p. amplitude at each [Ca] serves as the control for that run.

B. The time for decay of PTP to half amplitude as a function of external [Ca]. Recordings were obtained extracellularly from the end-plate region. The different symbols are the results of experiments on different muscles. The tetani were 62·5/sec for 20 sec.

as [Ca] was reduced. However, this apparent shift is complicated by two factors. The peak of potentiation may appear to occur later in higher [Ca] because of a larger superimposed post-tetanic depression. In addition, the absence of depression at low [Ca] may unmask facilitation which may then add to PTP at early intervals to produce an apparent maximum potentia-

tion near 'zero time'. Facilitation builds up during the train and should decay in two phases with time constants of approximately 35 and 350 msec (Mallart & Martin, 1967).

The question arises whether the effects of [Ca] on PTP are due to its presence during the tetanus or during the post-tetanic period. Experiments were performed in which low [Ca] solution was present during the train and normal [Ca] solution was present during the post-tetanic period. The recordings in all such experiments were made with intracellular micropipettes from single surface muscle fibres. In Fig. 6 the filled symbols in the upper graph show a control run in normal Ca solution. The tetanic stimulation of 62·5/sec for 20 sec is indicated by the bar. In the middle graph $\frac{1}{12}$ normal [Ca] solution was admitted to the bath during the time between the arrows (-2 to 0·5 min). The decline in amplitude of the e.p.p., tested with single stimuli at 30 sec intervals, is a result of the reduction of transmitter release at low [Ca], and the return to control values indicates the time course of recovery when normal [Ca] is readmitted to the chamber. In the lower record low [Ca] solution was again washed into the chamber during the interval between arrows. Two minutes after introducing the low [Ca] solution, an identical tetanic stimulation was given, and at 2·5 min normal [Ca] Ringer was readmitted to the bath. The filled symbols represent the recorded amplitudes, and the crosses the amplitudes corrected for the recovery of the e.p.p. from the low [Ca] wash. The rapid time course of PTP of the corrected amplitudes is similar to that seen in experiments performed entirely in low [Ca] solution (see Fig. 5*A*). It will be noted that by the time the potentiation in normal [Ca] is maximal (upper record, 3 min), there is no longer any potentiation in the trial in which low [Ca] was present during the train, although the control wash (middle) shows that the effects of low [Ca] on e.p.p. amplitude should have been reversed completely by this time. These experiments demonstrate that the effects of Ca depend on its presence during tetanic stimulation.

The presence of high levels of $MgCl_2$ (16 mM) in the bathing solution resulted in a PTP of brief time course, similar to that seen in solutions containing low [Ca]. This inhibition of the action of Ca can be compared with the inhibition by Mg of Ca-mediated transmitter release. In most experiments in Mg, intracellular recordings obtained from some fibres showed virtually no response for 1–3 min following tetanic stimulation and then responses at a potentiated or normal level. This suggests that block of conduction occurred in some nerve terminals for several minutes following a tetanus. This block is interpreted as being due to the combined effects of high external Mg in increasing nerve threshold and post-tetanic hyperpolarization of the terminals (Braun & Schmidt, 1966). This finding explains the fact that in this preparation, PTP usually appeared to be

absent in high Mg when the responses were recorded extracellularly from the whole muscle.

A second component of facilitation, which reaches a maximum about

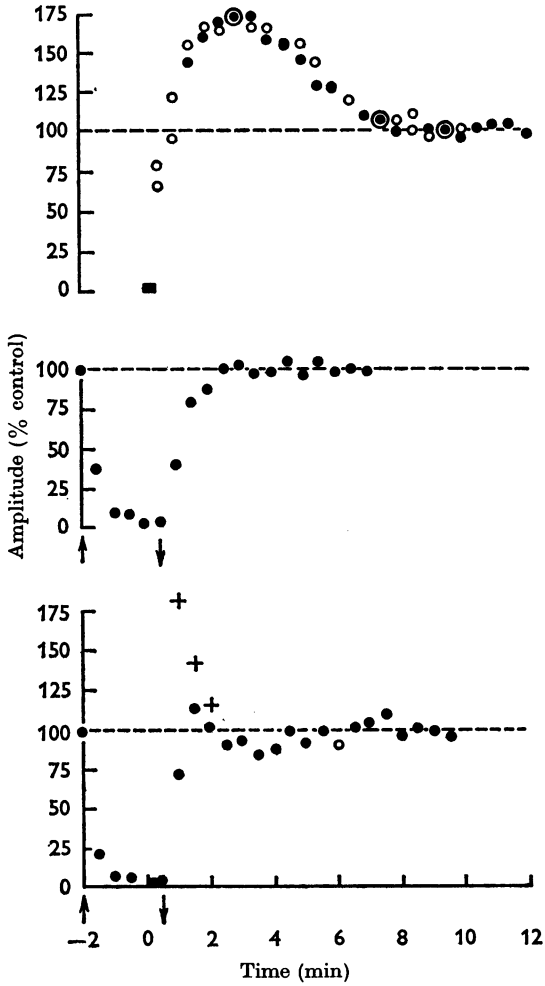

Fig. 6. The effect of low [Ca] during the tetanus. Recordings were obtained intracellularly from a surface muscle fibre. TC 4 μg/ml. Upper: variation of the e.p.p. amplitude following stimulation at 62·5/sec for 20 sec. Normal [Ca] (1·8 mM) was present throughout the runs. ● first run of series, ○ last run of series. Middle: changes in e.p.p. amplitude during wash in and wash out of $\frac{1}{12}$ × Ca (0·15 mM). The low [Ca] solution was present during the time between arrows. Lower: the effect of low [Ca] on PTP when present only during the tetanus. The wash in and wash out of low [Ca] is indicated by the arrows. The tetanus (62·5/sec for 20 sec) is indicated by the bar at zero time. The crosses are the values corrected for the recovery from the wash out.

100 msec after the end of a short train of 5 stimuli, and which decays with a time constant of about 350 msec, has been described by Mallart & Martin (1967). In $\frac{1}{6}$ normal Ca, short trains of 25 stimuli give rise to a period of potentiation lasting well beyond that of facilitation. It is not clear whether the second component of facilitation is identical with PTP, becoming more delayed and prolonged with increases in train duration, or whether it is a separate phenomenon.

DISCUSSION

The two major findings of this study are that PTP is quantitatively accounted for by an increase in the probability of release rather than an increase in available transmitter, and that PTP appears to be associated with an accumulation of Ca during the tetanus. The more rapid decline of a brief train of e.p.p.s during the post-tetanic period, as compared to a brief control train, has been cited previously by several workers as evidence that PTP is not due to an increase in the amount of available transmitter (Larrabee & Bronk, 1947; Liley & North, 1953; Elmqvist & Quastel, 1965). This study has extended those observations by demonstrating that the estimated changes in release probability, p, agree quantitatively in magnitude and time course with the e.p.p. amplitude changes during PTP.

The amplitude of PTP appeared to be relatively constant over a wide range of conditions (e.g. variation of the number of stimuli, [Ca] or temperature). The reason for this is not known, but it is not a result of an apparent saturation of the recorded response due to non-linear summation of e.p.p. amplitude, since the responses were less than 10 mV in intracellular recordings and equivalent to 1–2 mV in extracellular recordings.

It appears from several lines of evidence in the present study that PTP is most likely dependent on an accumulation of intracellular or membrane bound Ca during activity (cf. Gage & Hubbard, 1966). Those procedures which tend to increase Ca entry during a tetanus, such as increased number of stimuli or elevated external [Ca], also increase the time course of PTP. It is evident from the experiments in which low [Ca] solution was present only during the tetanus that PTP is dependent only on the external [Ca] present at the time of the tetanus. These experiments in particular eliminate the possibility that PTP is due to a post-tetanic state which allows increased Ca entry during the action potential resulting in increased transmitter release. The experiments which demonstrate the effects of Ca on PTP also suggest that changes in intracellular Na cannot be *directly* responsible for PTP. Presumably, the amount of Na entering the terminal during a tetanus is affected very little by changes in [Ca] which result in marked changes in the time course of PTP.

The mechanism which regulates intracellular [Ca] is largely unknown. The strong electrochemical gradient for Ca entry into the cell leads to the necessary hypothesis of a special mechanism for Ca extrusion. It may be that PTP is due to increased release of transmitter as a result of increased intracellular [Ca] following a tetanus, and that the time course of decay is determined by the time course of extrusion of this accumulated Ca. The extrusion may either involve a Ca pump or an exchange diffusion carrier, at which Na and Ca compete, of the type recently proposed in squid axon (Baker, Blaustein, Hodgkin & Steinhardt, 1967) and heart muscle (Reuter & Seitz, 1968). There is one major drawback to this proposal. If PTP is dependent on the accumulation of Ca during the tetanus, one would expect it to be maximal immediately after the cessation of stimulation when the accumulated [Ca] is greatest. However, the experiments which estimate the changes in p by the two shock method and experiments in the cold suggest that in normal [Ca] potentiation reaches its maximum amplitude only after some delay.

I am indebted to Dr A. R. Martin for many helpful discussions during the course of these experiments. This work was supported by U.S.P.H.S. Fellowship No. NB 32217 and Grant No. NB 07139.

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