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EFFECTS OF CALCIUM AND MAGNESIUM ON THE FREQUENCY OF MINIATURE END-PLATE POTENTIALS DURING PROLONGED TETANIZATION

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SUMMARY

1. End-plate potentials (e.p.p.s) and miniature end-plate potentials (min.e.p.p.s) were recorded intracellularly from the cutaneous pectoris nerve-muscle preparation of the frog during prolonged stimulation at low frequencies (5/sec–50/sec).

2. When Ca was present in the bathing solution, the quantum content of the e.p.p. and the frequency of occurrence of the min.e.p.p.s gradually increased during the period of stimulation. During the first few minutes of stimulation, the min.e.p.p. frequency increased linearly with time, and the rate of increase was dependent on the Ca concentration of the bathing solution. However, Mg had no effect on this Ca-dependent increase in min.e.p.p. frequency.

3. A large maintained increase in min.e.p.p. frequency also occurred during prolonged stimulation in solutions that contained no added Ca and 1–2 mM-EGTA. Under these conditions the increase in min.e.p.p. frequency was dependent on the Mg concentration of the bathing solution and was exponential in time.

4. It is suggested that the rise in min.e.p.p. frequency is caused by an accumulation of Ca or Mg ions in the nerve terminal, and it is suggested that these ions enter the terminal at relatively non-specific sites distinct from the Ca-specific sites that trigger the 'phasic' release of transmitter.

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INTRODUCTION

It is well known that tetanic stimulation of a motor nerve causes an in crease in the frequency of spontaneously occurring min.e.p.p.s at the neuro muscular junctions of mammals (Brooks, 1956; Liley, 1956*a*; Hubbard, 1963) and of frogs (del Castillo & Katz, 1954*b*; Braun, Schmidt & Zimmermann, 1966; Miledi & Thies, 1971). However, there seems to be disagreement among various workers about the Ca and Mg dependence of this post-tetanic discharge of min.e.p.p.s. On the one hand, Liley (1956*a*) noted that in rat diaphragm the post-tetanic increase in min.e.p.p. frequency was relatively insensitive to changes in the Ca or Mg concentration of the bathing solution, and Blioch, Glagoleva, Liberman & Nenashev (1968) have observed a Mg-dependent, post-tetanic, increase in min.e.p.p. frequency in frog muscles bathed in Ca-deficient solutions. On the other hand, del Castillo & Katz (1954*b*) reported that in frog muscle high concentrations of Mg ions suppressed the post-tetanic increase in min.e.p.p. frequency, and recently Miledi & Thies (1971) showed that this increase in min.e.p.p. frequency was dependent on Ca and they implied that it was inhibited by Mg. Although Miledi & Thies observed a post-tetanic increase in min.e.p.p. frequency in EGTA treated preparations, the increase was small, and these workers suggested that it may have been due to traces of Ca ions that persisted at the end-plate region even in the presence of EGTA (ethyleneglycol bis(aminoethylether)-N,N′-tetra-acetic acid).

In most of these earlier studies the tetani were brief (0·5–60 sec in duration) and the frequencies of stimulation were high (40–320/sec). We stimulated a neuromuscular preparation from the frog at relatively low frequencies (5–50/sec), for long periods of time (up to 4 hr) and found that the increase in min.e.p.p. frequency was affected by Ca and Mg ions in a rather complex manner. When preparations were stimulated in solutions that contained small concentrations of Ca ions (0·1–0·35 mM), the min.e.p.p. frequency increased linearly with time during the first few minutes of a tetanus. Under these conditions the rate of increase of the min.e.p.p. frequency was dependent on the Ca concentration, and was independent of the Mg concentration, of the bathing solution. When preparations were stimulated in solutions that contained no added Ca ions, the min.e.p.p. frequency built up approximately exponentially at a rate which increased with the Mg concentration of the bathing solution. EGTA had little effect on this build-up of the min.e.p.p. frequency.

Thus our results agree with those of Miledi & Thies (1971) in indicating that there is a Ca-dependent rise in min.e.p.p. frequency during a tetanus and they agree with those of Liley (1956*a*) and those of Blioch *et al.* (1968) in indicating that Mg ions are not a potent inhibitor of this rise. These

effects of Mg on the tetanically induced rise in min.e.p.p. frequency differ markedly from the effects of Mg on the e.p.p. or on the rise in min.e.p.p. frequency induced by depolarization of the nerve terminal. Magnesium is a potent inhibitor of these latter two modes of transmitter release (Liley, 1956*a*, *b*; del Castillo & Engbaeck, 1954; del Castillo & Katz, 1954*a*, *c*; Dodge & Rahamimoff, 1967; Landau, 1969).

The results presented here were obtained from the cutaneous pectoris muscle of the frog, but many of the phenomena were first observed in the sartorius muscle (Longenecker, 1970).

METHODS

Cutaneous pectoris muscles and their innervating nerves were dissected from frogs, *Rana pipiens*, and mounted in a lucite chamber. To facilitate the mounting, a bit of the rectus abdominus muscle was left attached to the caudal end of the cutaneous pectoris muscle and a small flap of the abdominal skin was left attached to the cephalic end. The nerve was dissected back to its union with the brachial nerve (for the relevant anatomy, see e.g. Blioch *et al.* (1968)). The muscle was stretched, dorsal surface up, over a small convex lucite lens on the bottom of the chamber and its ends were anchored in dental wax with small stainless-steel pins. The nerve passed through a slot in the wall of the muscle chamber and into two small wells separated by a slotted partition. These wells were filled with Ringer solution and each contained a platinum wire for stimulating the nerve. The slots through which the nerve passed were filled with Vaseline to reduce shunting. The nerves were stimulated by rectangular voltage pulses 0·1 msec in duration with an amplitude 3–4 times greater than threshold.

No special precautions were taken to oxygenate the solutions bathing the muscle. They were equilibrated with air and were changed intermittently by running in fresh solution from the barrel of a hypodermic syringe suspended above the muscle bath. Solution was removed from the chamber by continuous aspiration. The chamber held about 5 ml. of solution, and fresh solutions were run in at a rate of about 20 ml./min. When solutions were changed, the bath was flushed at least 3 times during a 10 min period and about 10 ml. of the new solution were used in each flush. Ten minutes was judged to be an adequate equilibration time since Ca-free solutions always abolished the e.p.p. in 3 min or less. The muscle was illuminated from below by diffuse light reflected off a piece of white filter paper and the preparation was observed with a dissecting microscope at magnifications of 15–60 ×. Junctional regions were impaled with glass micropipettes filled with 3 M-KCl. The resistances of the micropipettes ranged from 10 to 35 MΩ and the tip potentials were less than 10 mV. The electrodes were calomel half-cells filled with 3 M-KCl; the indifferent electrode was connected to the muscle bath via a salt bridge that contained 2 % agar and 120 mM-NaCl.

The recording amplifier was a differential DC amplifier with a gain of 100 and effective input resistance of 10^{10} Ω. The time constant of the recording system was about 0·4 msec when the resistance of the micropipette was 20 MΩ. The output of the amplifier was displayed on a double beam oscilloscope. One beam was a.c. coupled and was operated at high gain to record the e.p.p.s and the min.e.p.p.s; the other beam was d.c. coupled at low gain to record the resting potentials of the muscle fibres.

The face of the oscilloscope was photographed on moving film every few minutes and each photograph was 4–15 sec long. When the min.e.p.p. frequency approached 200/sec, single sweeps (sweep speeds of 5–20 msec/cm) were also photographed to facilitate counting the min.e.p.p.s. Ten e.p.p.s were measured on each record to determine the mean amplitude for that record. The amplitudes of up to ten min. e.p.p.s were also determined on each record. If the resting potential of the muscle fibre remained relatively constant (depolarization less than 10 mV) during an experiment, then the amplitudes of all the measured min.e.p.p.s were used to determine the mean amplitude of the min.e.p.p.s, and this single mean was used to compute the quantum contents of the averaged e.p.p.s. If the muscle fibre depolarized considerably (10 mV or more) during the experiment, the min.e.p.p.s were collected in groups and averaged, and each group corresponded to a 10 mV range of resting potential. No corrections were made for non-linear summation of the min.e.p.p.s (Martin, 1966).

When the min.e.p.p. frequency was low (about 1/sec or less) each record contained only a few min.e.p.p.s, so that only an approximate index of the rate could be obtained. When the min.e.p.p. frequency was moderate (above 10/sec), 50–100 min.e.p.p.s were counted on each record. When the frequency was above 100/sec, the number of min.e.p.p.s that occurred in 0·5–1·0 sec were counted. While these measurements are clearly of limited precision, accurate determinations were unnecessary since the amplitude of the e.p.p.s changed about twentyfold during the experiments and the min.e.p.p. frequencies increased by two orders of magnitude.

Solutions. The standard Ringer solution contained 114 mM-NaCl, 2·1 mM-KCl, 1·8 mM-$CaCl_2$, 1 mM-NaH_2PO_4, 2 mM-Na_2HPO_4 and the pH was 7·0. When the ionic composition of this solution was varied, the tonicity was held constant by adjusting appropriately the concentration of the NaCl. Since solutions deficient in both Ca and Mg are deleterious to nerve and muscle (Bülbring, Hollman & Lüllman, 1956; Curtis, 1963; Jenden & Reger, 1963; Frankenhauser, 1957), we added Mg to all the low-Ca solutions. In our initial experiments 4 mM-Mg was used since this concentration of Mg has roughly the same effect on nerve excitability as does the Ringer concentration of Ca (Frankenhauser & Meves, 1958).

The EGTA was dissolved in an excess of 1 N-NaOH and the resulting solution was titrated with 0·1 N-HCl to a pH 7·1–7·2. This solution was diluted with water and sufficient NaCl was added to give a stock solution of the following composition: Na^+ = 140 mM, Cl^- = 100 mM and $EGTA^{-2}$ = 20 mM. This stock solution was diluted to provide solutions with 1 or 2 mM-EGTA.

Resting potentials. We had considerable difficulty recording steady resting potentials from cutaneous pectoris muscles soaking in Ca-deficient solutions. The membrane potentials ranged between 80 and 100 mV when the muscle fibres were first penetrated, but in most of the early preparations this potential gradually declined to 50 mV or less over the course of several hours. This decline in membrane potential was not due to a direct effect of the Ca-deficient solutions on the muscle fibres, but seemed to be caused by improper sealing of the micropipette into the fibres. Whenever fibres were freshly impaled, regardless of how long they had been soaking in the Ca-deficient media, the membrane potentials were in the normal range; the depolarization occurred only when the micropipette was left in a single fibre for a long period of time. We found that many depolarized fibres would repolarize to nearly normal levels if they were soaked for ½–1 hr in Ringer solution, and sometimes this soaking in Ringer gave a lasting seal so that depolarization did not occur when the Ca-deficient solutions were introduced again. Ultimately, the following working procedure was adopted. Neuromuscular transmission was blocked by bathing the preparation in a solution with 4 mM-Mg and 0·5–0·7 mM-Ca. A fibre was impaled in

the junctional region and a check made for the presence of e.p.p.s and min.e.p.p.s. If these were satisfactory (e.p.p. rise time of about 2 msec), then Ringer was run into the chamber and the preparation was left for ½–1 hr. If the resting potential had not declined by more than 10 mV during this time, an experiment was usually begun. When this routine was followed, the resting potentials were much more stable, although depolarization usually occurred in Ca-deficient solutions, especially when EGTA was used. As a given fibre depolarized, the min.e.p.p.s became progressively smaller, but observations were continued as long as the min.e.p.p.s could be readily distinguished from the noise level of the recording system. This usually occurred at membrane potentials of 40–50 mV. Between successive periods of stimulation, the preparations were usually soaked in Ringer solution for about a half hour to permit repolarization and to insure full recovery from the previous tetanus.

Ca determinations. A modification of the method described by Williams & Moser (1953) was used to determine the Ca content of our Ca-deficient solutions, and we obtained results similar to those of Miledi & Thies (1971). The Ca concentration of nominally Ca-free solutions ranged from 10–30 μM before the solutions were applied to the muscles, and they ranged from 40 to 50 μM after having been applied to the muscles for about half an hour.

RESULTS

Effect of prolonged stimulation without added Ca. Fig. 1 shows the time course of the changes in min.e.p.p. frequency during an experiment in which the nerve was stimulated continuously for 110 min at 10/sec in a solution with 4 mM-Mg and no added Ca. During the period of stimulation the min.e.p.p. frequency rose gradually and after about 40 min had reached rates of 200–300/sec. This high rate of min.e.p.p. discharge was well maintained during the next 60 min of stimulation, and when the stimulation ceased, the min.e.p.p. frequency fell to control levels in about half an hour. We performed many experiments of this kind. The steady-state rates of min.e.p.p. discharge ranged from about 120/sec to about 260/sec, and the times required for the min.e.p.p. frequencies to rise to half of these steady levels ranged from 10 to 40 min. In some experiments the min.e.p.p. frequencies began to fall off after about an hour of stimulation (this seems to occur in Fig. 1), and this may have been due to conduction failure in the nerve terminals. When failing preparations were rested for about an hour, it was usually possible to stimulate them again and to evoke another high frequency discharge of min.e.p.p.s. In many of the experiments the min.e.p.p. amplitudes fell, but this fall was probably due to the decline in the membrane potential of the muscle fibre. In other experiments with finite concentrations of Ca, in which the muscle membrane potentials did not decline, the min.e.p.p. amplitudes fell by less than 20 %. These results are similar to those of Elmqvist & Quastel (1965) with rat diaphragm.

Two observations indicate that the rise in the min.e.p.p. frequency was not caused by a rise in the Ca concentration of the bathing solution. First, the min.e.p.p. frequency did not change appreciably when fresh Ca-

deficient solution was admitted into the chamber. Secondly, in six experiments a CAT computer was used to see if there were any responses linked in time to the stimulus. In only one of these experiments was there an indication of an average response, and in this case the average response, determined for 100 shocks, was about 0·2 mV, or about half the amplitude of an individual min.e.p.p. The min.e.p.p. frequency at this time, determined from film, was 100–200/sec. The amplitude of the average response may not be a very sensitive measure of the ambient concentration of Ca when this concentration is low because the stimulus evoked release of

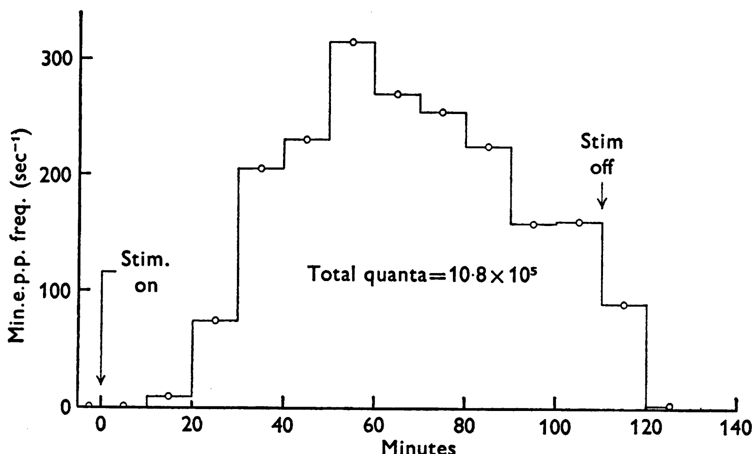

Fig. 1. Time course of the changes in the min.e.p.p. frequency during continuous stimulation at 10/sec in solution with no added Ca and 4 mM-Mg. Ordinate: frequency of occurrence of min.e.p.p.s. Abscissa: time in minutes. The membrane potential of this fibre declined from 75 to 50 mV during the period of stimulation. The min.e.p.p. frequency in the resting preparation was about 0·3/sec.

transmitter becomes a rare event under these conditions. Thus, although we cannot be sure that there was no Ca around the neuromuscular junctions in our experiments and that no e.p.p.s occurred, it appears that the enhanced rate of spontaneous discharge of min.e.p.p.s was less dependent on extracellular Ca than was the e.p.p.

Effect of frequency of stimulation. Since there was no previous report of such large increases in the min.e.p.p. frequency at such low rates of stimulation in solutions of such low Ca content, we examined these changes in more detail. We first studied the effect of the frequency of stimulation on the rate of increase in the min.e.p.p. frequency. Fig. 2*A* shows the averaged results obtained during tetanizations at frequencies of 5/sec, 10/sec, 20/sec and 50/sec. At least two, often three, different frequencies of stimulation were tested on each preparation with 10/sec serving as a control frequency.

The preparations were allowed to rest for at least a half hour between successive bouts of stimulation. With this interval of rest, successive bouts of activity at the same frequency of stimulation gave reproducible results.

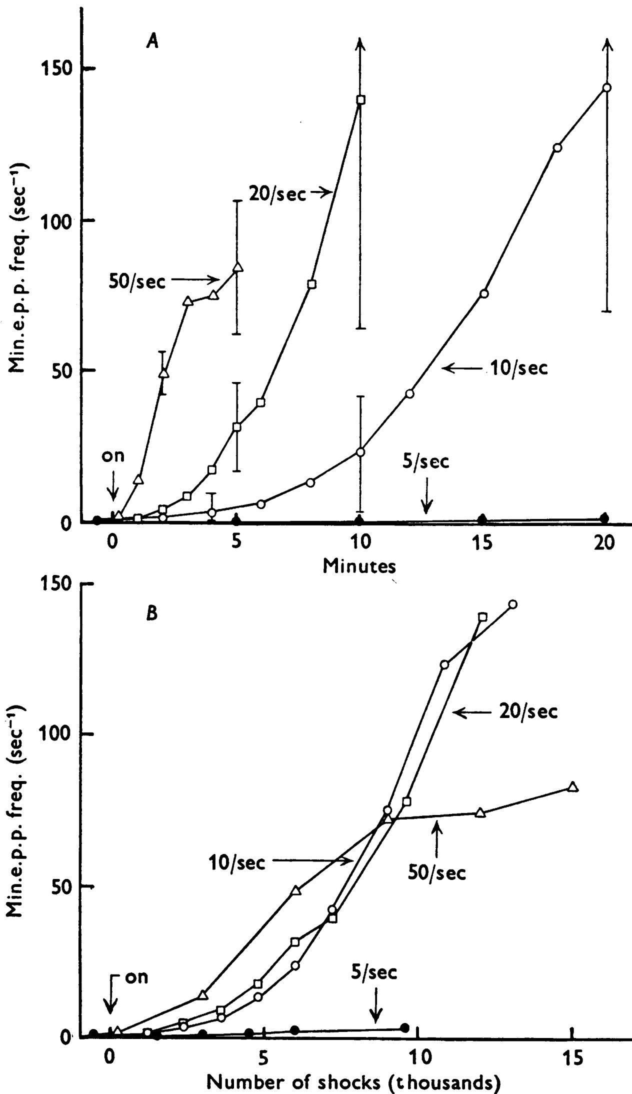

Fig. 2. Effect of frequency of stimulation on the increase in min.e.p.p. frequency during tetanization in solutions with no added Ca and 4 mM-Mg. Averaged results of all experiments. (*A* : ordinates and abscissae as in Fig. 1.) Bars indicate spread of data which was off scale for the highest values at 20/sec and 10/sec. Number of tests at each frequency were: 2 at 50/sec, 4 at 20/sec, 11 at 10/sec, 4 at 5/sec. Mean min.e.p.p. frequency in resting preparations was about 0·7/sec.

In Fig. 2*B* the data are plotted against the total number of shocks applied to the nerve. It is clear that the build-up of the min.e.p.p. frequency was determined primarily by the number of shocks applied to the nerve and was relatively independent of the frequency at which they were applied, provided the frequency was greater than 5/sec. The falling off of the min.e.p.p. frequency that occurred after 3 min of stimulation at 50/sec was probably due to conduction failure in the nerve terminals (Krnjević & Miledi, 1959).

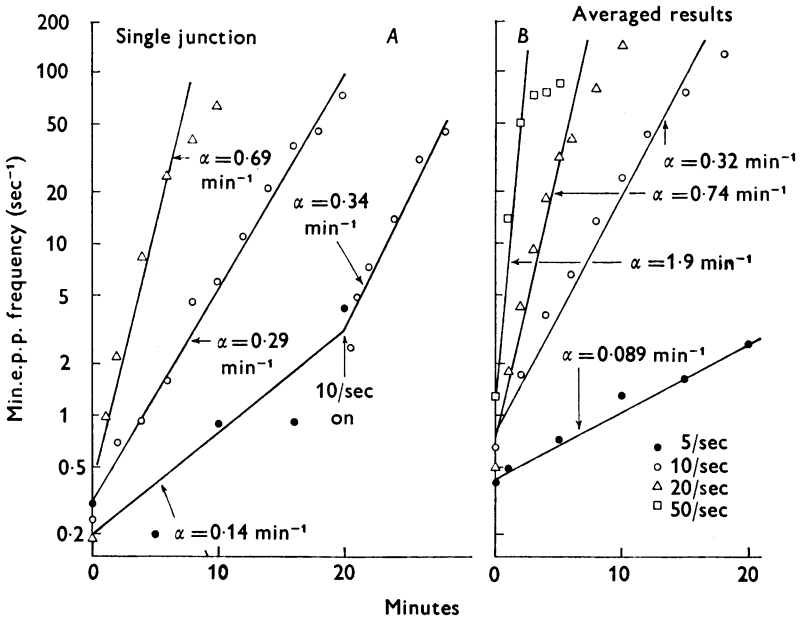

Fig. 3. Effect of frequency of stimulation on the increase in min.e.p.p. frequency during tetanization in a solution with no added Ca and 4 mM-Mg. Ordinates: min.e.p.p. frequency on a logarithmic scale. Abscissae: time in minutes after beginning stimulation. *A*: single junction. *B*: average results, the same data as in Fig. 2*A*. Lines drawn by eye. The α's indicate the values of the rate constants calculated for the various straight lines. In *A* the order of the tests was: 20/sec, 10/sec, 5/sec. After 20 min of stimulation at 5/sec, the frequency was raised to 10/sec. The membrane potentials during the tests were: 45–62 mV at 20/sec, 52–58 mV at 10/sec; 60–70 mV at 5/sec.

Fig. 3 shows that at a fixed frequency of stimulation the increase in min.e.p.p. frequency was approximately exponential in time, and it is clear that the exponential build-up began immediately the stimulation was begun. The apparent delays in the increase in the min.e.p.p. frequency that are evident in Figs. 1 and 2 are consequences of this exponential relation between the min.e.p.p. frequency and time. Since the rate constant of the

exponential increase was roughly proportional to the frequency of stimulation, if this frequency was 10/sec or more, the results can be conveniently summarized by the statement that under these conditions of stimulation the min.e.p.p. frequency was exponentially related to the total number of shocks applied to the nerve.

Although the min.e.p.p. frequency did not rise appreciably during stimulation at 5/sec, the terminal was conditioned by such stimulation. Thus, whenever the frequency of stimulation was suddenly increased to 10/sec after a prolonged period at 5/sec, the min.e.p.p. frequency rose much more rapidly than when the fully rested preparation was stimulated at 10/sec. This conditioning also is a consequence of the exponential time course of the rise in the min.e.p.p. frequency, and is illustrated in the lowermost curve of Fig. 3*A*. When the rate of stimulation was increased from 5/sec to 10/sec, the rate of increase in the min.e.p.p. frequency changed immediately. The increase was always exponential in time, but the rate constant of the exponential shifted abruptly to the value appropriate for 10/sec when the rate of stimulation was increased. The absolute rate of increase of the min.e.p.p. frequency was greater in the conditioned state than in the rested state simply because in the conditioned state the min.e.p.p. frequency had been increased slightly above the resting level by the previous stimulation at 5/sec.

The pattern of relaxation of the min.e.p.p. frequency following a tetanus was highly variable. When the tetanus was relatively brief (about 10 min in duration), the decline in the min.e.p.p. frequency was roughly exponential in time. However, when the duration of the tetanus was 20 min or more the decline in frequency was seldom exponential. Then either the frequency fell almost linearly, or the frequency declined slowly for the first 3 or 4 min and then fell rapidly to the control levels. The delay in the relaxation of the min.e.p.p. frequency was especially marked after prolonged periods of stimulation. In these cases the min.e.p.p. frequency often remained nearly constant for up to 5 min after stimulation had ceased before it began to decline. Occasionally, the min.e.p.p. frequency did not fall after the stimulation of the nerve had ceased. This occurred most commonly after a junction had been subject to several previous bouts of activity. Such cases have been excluded from the present results. In most cases (twelve of thirteen) where the duration of stimulation was 30 min or less, the min.e.p.p. frequency had decreased by 50 % within 2–5 min after stimulation had ceased, and in eleven of these cases the frequency had fallen below 5/sec about 10 min later.

Effect of EGTA. Physiological salt solutions to which no Ca has been intentionally added contain Ca at a concentration of about 0·03 mM (Miledi & Thies, 1971; and Methods). The level of ionized Ca can be reduced

to about 10^{-5} mM by adding 1 mM-EGTA to such a solution (Miledi & Thies, 1971; Hubbard, Jones & Landau, 1968). To rule out the possibility that traces of Ca were responsible for the observed increases in the min. e.p.p. frequency, we conducted some experiments in solutions that contained 1 mM-EGTA. The results of these experiments are shown in Fig. 4. In these experiments two tests were run on each neuromuscular junction: one test was carried out in the Ca-deficient solution and the other in the Ca-deficient solution with EGTA. For junctions *A*, *B* and *C* the test with EGTA was conducted after the test without EGTA, but for junction *D*

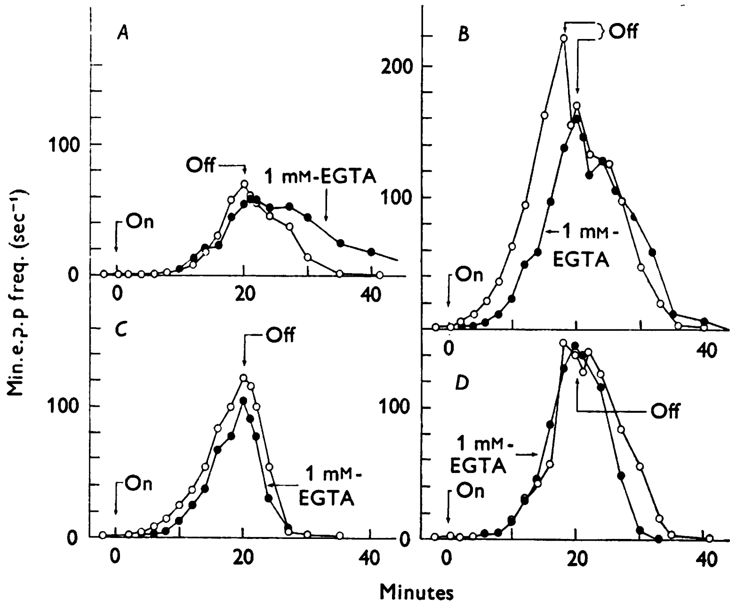

Fig. 4. Effect of 1 mM-EGTA on the increase in min.e.p.p. frequency during tetanization in solutions containing no added Ca and 4 mM-Mg. Ordinate and abscissa as in Fig. 1. Frequency of stimulation, 10/sec. Membrane potentials were: 55–65 mV in *A*, 70–80 mV in *B*, 40–80 mV in *C*, 50–85 mV in *D*. Mean min.e.p.p. frequencies: 0·5/sec in 0-Ca; 0·3/sec in 1 mM-EGTA.

the test with EGTA was conducted first. It is clear that stimulation provoked a large increase in the min.e.p.p. frequency in the EGTA solutions. The min.e.p.p. frequency rose a little more slowly in the solutions that contained EGTA than in those that did not, and this indicates that the traces of Ca present in the latter solutions had a small, but not crucial, effect on the rate of increase in the min.e.p.p. frequency.

Effect of Mg in the absence of Ca. Blioch *et al.* (1968) have suggested that any multivalent cation that enters a nerve terminal can elevate the min. e.p.p. frequency, and they reported that, in Ca-deficient solutions, Mg

accelerated the post-tetanic discharge of min.e.p.p.s. Therefore, we studied the effects of Mg on the increase in the min.e.p.p. frequency that occurs during stimulation in solutions with no added Ca. The results from one experiment are shown in Fig. 5 and the averaged results from all the experiments are shown in Fig. 6.

Fig. 5 shows the results obtained from a single junction on which we tested the effects of 2 mM and 10 mM-Mg with and without EGTA. The calculations of Hubbard *et al.* (1968) indicate that in our solutions about

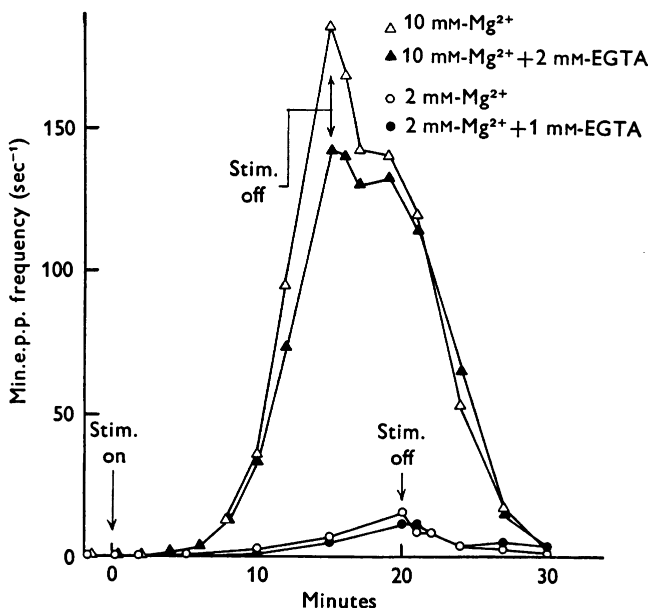

Fig. 5. Effect of Mg on the increase in min.e.p.p. frequency during stimulation at 10/sec in solutions with no added Ca. Ordinate and abscissa as in Fig. 1. Single junction. Order of tests was: 2 mM-Mg, 2 mM-Mg + 1 mM-EGTA, 10 mM-Mg + 2 mM-EGTA, 10 mM-Mg. Membrane potentials were: 70–80 mV in 2 mM-Mg; 50–70 mV in 2 mM-Mg + 1 mM-EGTA; 30–50 mV in 10 mM-Mg + 2 mM-EGTA; 45–55 mV in 10 mM-Mg. Resting min.e.p.p. frequency was 0·1–0·2/sec.

10 % of the Mg is chelated by EGTA. Therefore, more EGTA was added to the solution with 10 mM-Mg so that the concentration of free EGTA, that is the amount of EGTA not complexed by Mg, would be approximately the same in this solution as in the solution with 2 mM-Mg. We estimated the concentration of free EGTA to be 0·8–1 mM in this experiment. It is clear that the min.e.p.p. frequency rose much more rapidly in the solutions with the higher Mg concentrations. Since EGTA had little effect on this rise in the min.e.p.p. frequency, it seems likely that the effect was due directly to Mg and was not due to traces of Ca present in the Mg solutions.

With any given concentration of Mg, the min.e.p.p. frequencies varied considerably from preparation to preparation. However, averaging the results showed the over-all effect to be smoothly graded (Fig. 6). When the data in Fig. 6 were plotted on semilogarithmic graph paper it was clear that the main effect of Mg was on the rate constant of the exponential increase in min.e.p.p. frequency. This effect of Mg is illustrated in Fig. 7.

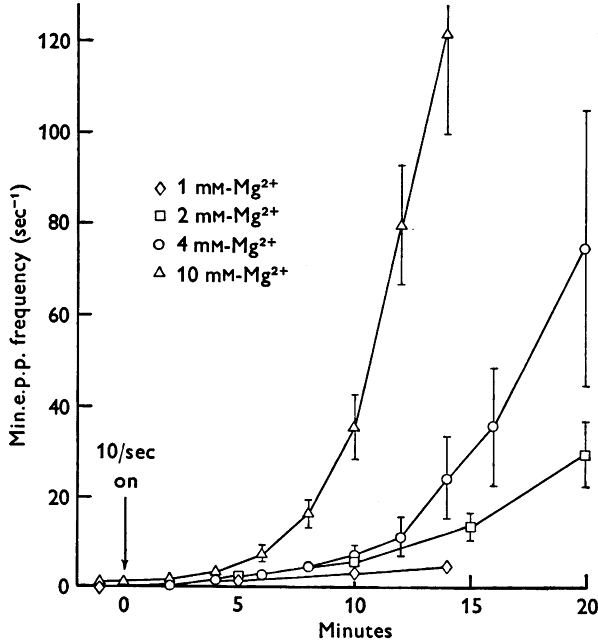

Fig. 6. Effect of Mg on the increase in min.e.p.p. frequency during stimulation at 10/sec in solutions with no added Ca. Ordinate and abscissa as in Fig. 1. Averaged results. Bars indicate ±1 S.E. of the mean. Number of tests at the various concentrations of Mg were: five at 1 mM, six at 2 mM, six at 4 mM and ten at 10 mM. Whenever several different concentrations of Mg were tested in the same preparation, the magnitudes of the increases in min.e.p.p. frequency were always ranked as shown above. Mean values of the min.e.p.p. frequencies in resting preparations were: 0·4/sec in 1 mM-Mg, 0·4/sec in 2 mM-Mg, 0·5/sec in 4 mM-Mg and 1·0/sec in 10 mM-Mg.

The relation between the rate constant and the Mg concentration was non-linear and seemed to obey Michaelis-Menton kinetics with a K_m of about 2 mM and an α_{max} of about 0·5 min^{-1}.

We did four experiments with concentrations of Mg between 12 and 20 mM. In two of these experiments the min.e.p.p. frequency rose even more rapidly than in experiments with 10 mM-Mg. In the other two experiments the min.e.p.p. frequency did not rise at all during the period of stimulation, and we assumed that in these cases conduction failure

occurred in the nerve terminals. Concentrations of Mg greater than 10 mM were thereafter avoided.

Fig. 7 also illustrates how rapidly the min.e.p.p. frequency changed in response to step changes in the Mg concentration of the bath during con-

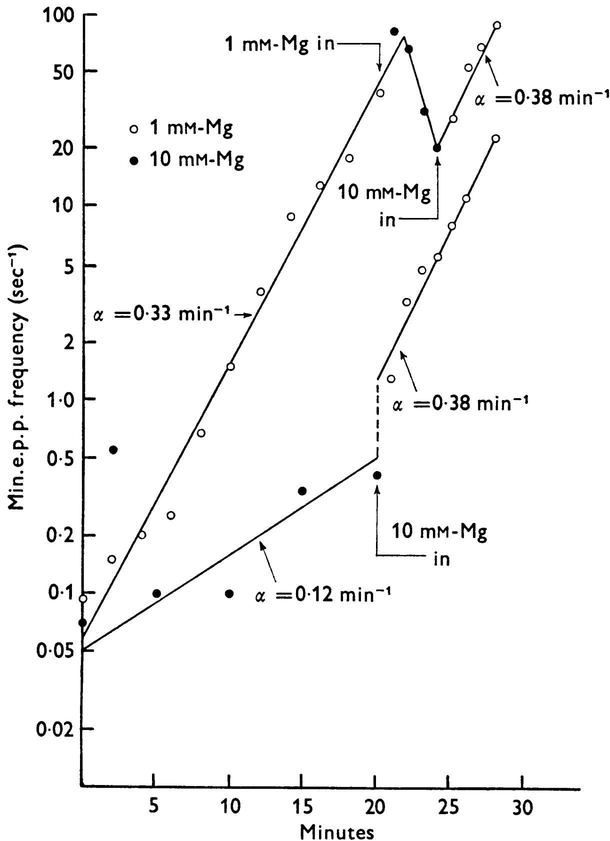

Fig. 7. Effect on min.e.p.p. frequency of changing the concentration of Mg in the bath during continuous stimulation at 10/sec. Ordinates and abscissae as in Fig. 3. Single junction. Lines drawn by eye. The arrows indicate when the bathing solution was changed. Membrane potential: upper curve, 77–56 mV; lower curve, 85–78 mV. Min.e.p.p. frequency in the resting preparation was about 0·05/sec. Although normal in other respects this junction had an unusually low resting min.e.p.p. frequency, and because of this we were able to stimulate a relatively long time without elevating this frequency to levels that precluded accurate counting.

tinuous stimulation. It is clear that step changes in the Mg concentration did not produce step changes in the min.e.p.p. frequency. When the Mg concentration was changed abruptly, the slopes of the lines in Fig. 7 changed abruptly from one value to another, while the min.e.p.p. fre-

quency changed progressively at rates governed by the new slopes. This indicates that in tetanized preparations the min.e.p.p. frequency is not directly determined by the extracellular concentration of Mg as seems to be the case in depolarized terminals (Liley, 1956*b*; del Castillo & Katz, 1954*c*; Hubbard, 1961; Landau, 1969).

The rates of change of the min.e.p.p. frequency were not limited by the rate of diffusion of Mg into the junctional regions. This was checked by raising the Mg concentration in K-rich solutions and determining the subsequent changes in the min.e.p.p. frequency. In solutions with 10 mM-K and 1·8 mM-Ca the min.e.p.p. frequency ranged from 100 to 400/sec. When the Mg concentrations of these solutions were suddenly raised to 10 mM, the min.e.p.p. frequencies fell to 2–8/sec within 2 min. These data show that Mg affected the K-induced increase in min.e.p.p. frequency in a conventional manner, and that the action was rapid. Thus, the effects of Mg on the tetanized terminals differed markedly from the effects on depolarized terminals both as to the direction of the effect and as to the speed of action.

Effect of Mg in the presence of Ca. Since these effects of Mg in Ca-deficient solutions were somewhat unusual, we decided to examine the effects of Mg in the presence of Ca. In each of these experiments we examined on a single junction the effect of 2, 4 and 10 mM-Mg in the presence of 0·25 mM-Ca. The averaged results are shown in Fig. 8. We found no obvious effects of Mg on the build-up of the min.e.p.p. frequency during the initial stages of the tetani and for this reason all of the results were combined to get the upper curve in Fig. 8*B*. The results may be summarized as follows.

(*a*) During a tetanus the min.e.p.p. frequency rises more rapidly in the presence of Ca than in its absence. In the presence of Ca the rise in frequency begins immediately and proceeds linearly in time for a few minutes. Thus, part of the rise in the min.e.p.p. frequency is Ca-dependent.

(*b*) The initial rate of rise of the min.e.p.p. frequency is independent of the concentration of Mg. This lack of effect of Mg on the Ca-dependent component of the rise in the min.e.p.p. frequency contrasts strongly with the strong inhibitory effect of Mg on the quantum content of the e.p.p. (Fig. 8*A*).

(*c*) The time course of the relaxation of the min.e.p.p. frequency was affected by Ca. When stimulation was stopped, and Ca had been present, the min.e.p.p. frequency fell rapidly and within a minute reached a level close what would have been reached had no Ca been present. The subsequent relaxation followed a curve characteristic of Ca-deficient solutions.

The slowly developing potentiation of the e.p.p. shown in Fig. 8*A* was described previously by Braun *et al.* (1966), and is clearly different from the short-lived potentiation studied by Mallart & Martin (1967). As did

Braun *et al.* (1966), we found that during more prolonged stimulation the e.p.p. would reach a peak after 20–30 min and then decline to almost zero after about an hour while the min.e.p.p. frequency remained high.

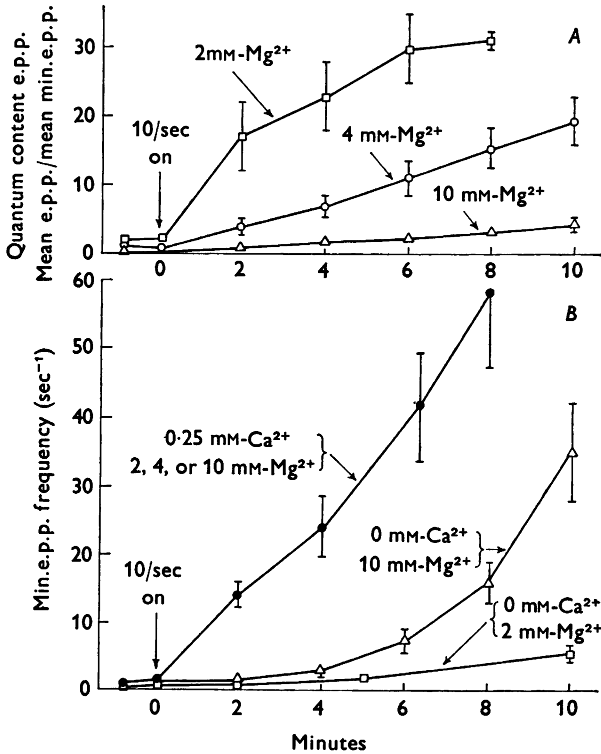

Fig. 8. Effect of Mg on the quantum content of the e.p.p. and on the min.e.p.p. frequency during tetanization in solutions with 0·25 mM-Ca. *A*: quantum content of e.p.p. *B*: min.e.p.p. frequency. Frequency of stimulation 10/sec. Average values of three experiments, each with 2, 4 and 10 mM-Mg. Bars indicate ± 1 S.E. of the mean. Since Mg had little effect on the initial rate of increase in min. e.p.p. frequency, all the results were combined into a single curve in *B* so that this curve is an average of nine tests. The other two curves in *B* (0 mM-Ca + 2 mM-Mg and 0 mM-Ca + 10 mM-Mg) were taken from Fig. 6. The mean values for the quantum contents of the e.p.p.s in resting preparations at the various levels of Mg were: 1·7 in 2 mM-Mg, 0·45 in 4 mM-Mg, and 0·1 in 10 mM-Mg. The mean min. e.p.p. frequency in these resting preparations was about 1·5/sec.

Fig. 9*A* summarizes the average results obtained when the Ca concentration of the bathing solution was varied while the Mg concentration was fixed. In Fig. 9*B* the increase in the min.e.p.p. frequency produced by 2000 shocks is plotted against the logarithm of the ratio of the Ca concentration to the Mg concentration of the bathing solution. This kind of

plot was used to facilitate comparing our data with those of Miledi & Thies (1971) who carried out a similar study on frog sartorius muscle, but used briefer periods of stimulation (about 20 sec) and higher frequencies (50–100/sec). There is good agreement between our results and theirs, and it seems that both groups have studied the same Ca-dependent process. However, we feel that a plot such as Fig. 9*B* could convey a misleading impression since it implies that Mg has an inhibitory effect on the Ca-dependent,

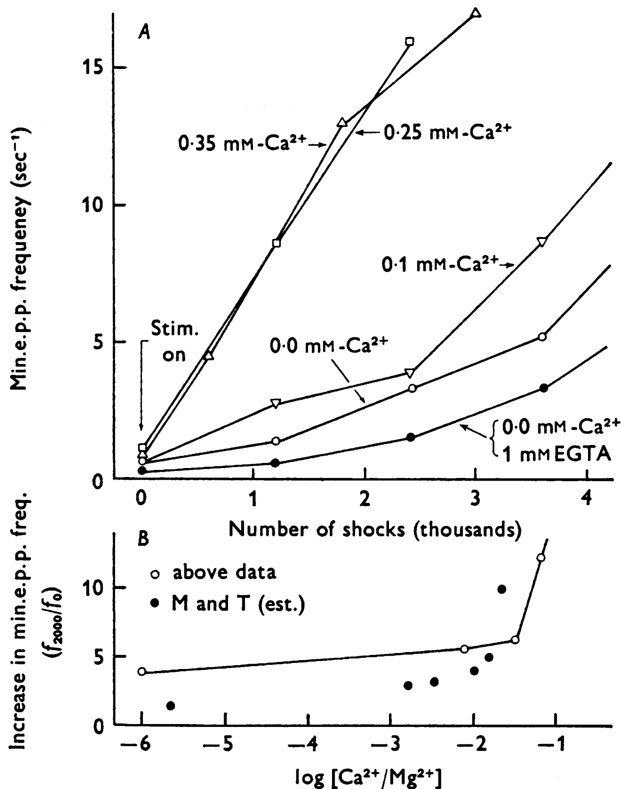

Fig. 9. Effect of Ca on the increase in min.e.p.p. frequency during tetanization at 10/sec in solutions with 4 mM-Mg. *A*: average values from present experiments. *B*: comparison with results of Miledi & Thies (1971). Ordinates: *A*, frequency of occurrence of min.e.p.p.s; *B*, ratio of min.e.p.p. frequency after 2000 shocks to min.e.p.p. frequency in resting preparation. Abscissae: *A*, number of shocks delivered to nerve; *B*, logarithm of the ratio of the Ca to the Mg concentration in the bathing solution. In *A* the number of experiments at the various concentrations of Ca were: four in 1 mM-EGTA, eleven in 0 mM-Ca, three in 0·1 mM-Ca, three in 0·25 mM-Ca, four in 0·35 mM-Ca. To make part *B* we assumed, after Miledi & Thies (1971), that in solutions with no added Ca, the Ca concentration was 0·03 mM and that in solutions with 1 mM-EGTA, the Ca concentration was 4×10^{-9} M.

tetanically induced rise in the min.e.p.p. frequency, whereas we have found that Mg has little effect on this rise in the min.e.p.p. frequency.

These experiments with solutions containing both Ca and Mg provide additional evidence against the proposition that traces of Ca are responsible for the rise in the min.e.p.p. frequency that occurs during tetanization in nominally Ca-free solutions. This is seen if one compares the results obtained in a solution containing 10 mM-Mg and no added Ca with the results obtained in solutions containing 1 or 4 mM-Mg and 0·1 mM-Ca. In the former solution after 12 min of stimulation at 10/sec the average min. e.p.p. frequency was 79 ± 13/sec ($\pm$ S.E. of mean, $N = 10$), and in the latter solutions the average min.e.p.p. frequency was 29 ± 11/sec ($\pm$ S.E. of mean, $N = 5$). This difference is significant at the 1 % level (*t* test). Unfortunately, with one exception, these tests were not made on the same junctions. In this case we found that after 14 min of stimulation in a solution containing 10 mM-Mg and no added Ca the min.e.p.p. frequency was 42/sec and there were no obvious e.p.p.s; after 14 min of stimulation in a solution with 1 mM-Mg and 0·1 mM-Ca the min.e.p.p. frequency was only 11/sec and the quantum content of the e.p.p. was about 5. Thus, both this single paired result and the averaged results indicate that the effect of 10 mM-Mg on the min.e.p.p. frequency was greater than the effect of 0·1 mM-Ca, and it seems likely that the effects obtained with the high concentration of Mg were due to the Mg *per se* and were not due to traces of Ca.

DISCUSSION

We can readily exclude two possible explanations for the enormous rise in min.e.p.p. frequency that occurred when our preparations were tetanized in Ca-deficient solutions. These are:

(1) that the rise is due to a depolarization of the nerve terminal, and

(2) that the rise is due to a direct stimulatory effect of extracellular Mg on the transmitter release sites in the nerve terminals.

It seems unlikely that the rise in the min.e.p.p. frequency during tetanization is caused by a depolarization of the nerve terminal. The increase in the min.e.p.p. frequency that is induced by depolarization requires extracellular Ca and is inhibited by Mg (Liley, 1956*b*; del Castillo & Katz, 1954*c*; Hubbard, 1961; Landau, 1969). We find that the increase in the min.e.p.p. frequency induced by tetanization occurs in the virtual absence of extracellular Ca and under such conditions seems to require extracellular Mg. The requirement for Mg does not mean that the extracellular Mg acts directly on the release sites to induce the spontaneous release of transmitter as appears to be the case in unstimulated terminals of the rat (Hubbard *et al.* 1968). In the tetanized terminals of the frog the effect of

extracellular Mg on the min.e.p.p. frequency seems to be indirect and to arise secondarily as a consequence of other Mg-dependent changes in the state of the nerve terminal that are induced by the tetanization. This is indicated by the results of experiments in which the Mg concentration in the bath is raised suddenly during a tetanus. In such experiments the increase in the min.e.p.p. frequency occurs too slowly to be ascribed to a direct stimulation of the release sites by the added Mg.

We cannot exclude categorically the possibility that our results are due to the presence of trace amounts of Ca in the junctional regions, for the Ca hypothesis of Katz & Miledi (1968, 1970) can be modified to account for enhanced spontaneous rates of min.e.p.p. discharge ('residual' release of transmitter) in the virtual absence of e.p.p.s ('phasic' release of transmitter) (Miledi & Thies, 1971). Thus, in their experiments with EGTA solutions Miledi & Thies (1971) found that the min.e.p.p. frequency rose to about 2/sec after the nerve had been stimulated 2000 times. They detected no evoked responses under these conditions. They calculated, with suitable assumptions, that these results would be explained if the Ca concentration around the release sites were about $1\cdot5 \times 10^{-8}$ M rather than 4×10^{-9} M, the concentration estimated from the binding constant of EGTA to be present in the solutions. We observed min.e.p.p. frequencies of 100–200/sec after the nerve had been stimulated by 12,000 shocks and we, too, saw no evoked responses. Our results are consistent with their hypothesis if one assumes that the min.e.p.p. frequency increases with the fourth power of the number of shocks. Thus it appears that, if the assumptions used by Miledi & Thies (1971) are valid, our results could be accounted for by traces of Ca persisting in the junctional regions even in the presence of EGTA. However, we prefer not to adopt this hypothesis. Our main evidence against it is the finding that the rise in the min.e.p.p. frequency requires Mg. This requirement for Mg is genuine and cannot be attributed to traces of Ca contaminating the Mg solutions. This is indicated by our other finding that the stimulatory effects of high concentrations of Mg cannot be matched by concentrations of Ca as great as 0·1 mM. An alternative possibility is that the rise in the min.e.p.p. frequency induced by stimulation in Ca-deficient solutions is truly independent of extracellular Ca.

A possible explanation for the apparent effect of Mg on the min.e.p.p. frequency is that partial block develops in the nerve terminals bathed in Ca-deficient solutions with little Mg (Krnjević & Miledi, 1959) so that not every action potential propagates to the end of the nerve. If raising the Mg concentration increased the proportion of action potentials reaching the nerve endings, then the higher min.e.p.p. frequencies seen at the higher concentrations of Mg might be accounted for. Although partial nerve block may account for some of the effect of Mg, it cannot account for all of it.

Thus, after about 14 min of stimulation in solutions with 0·1 mM-Ca and 1 mM-Mg we observed an e.p.p. in response to every stimulus; the mean quantum content of the e.p.p. was about five in these experiments. This indicates that there was no nerve block in these solutions with small concentrations of Ca, yet the min.e.p.p. frequency was much less in these experiments than in other experiments in which solutions with 10 mM-Mg and no added Ca were used (p. 33). Of course this does not prove that partial block does not occur in the absence of Ca, but it does show that the effects of the higher concentrations of Mg cannot be entirely due to the prevention of intermittent conduction block.

We have no simple model to account for our results but many of our findings, especially the dependence of the min.e.p.p. frequency on the total number of shocks applied to the nerve, suggest that the rise in the min.e.p.p. frequency is due to the temporal summation of some effect produced by the individual action potentials. Ca and Mg ions move into nerve fibres during stimulation (Hodgkin & Keynes, 1957; Baker & Crawford, 1971), so it seems reasonable to suggest that Ca and Mg accumulate in the nerve terminals during a tetanus and that the spontaneous min.e.p.p. frequency is controlled by the concentration of these ions within the terminal, as suggested by Blioch *et al.* (1968).

The ineffectiveness of Mg as an inhibitor of the Ca-dependent rise in the min.e.p.p. frequency might be explained if there were two routes by which Ca could enter the nerve terminal. One of the routes of Ca entry is that proposed by Katz & Miledi (1968, 1970) to provide the Ca required for the phasic release of transmitter. It is relatively specific for Ca, is inhibited by Mg (Blaustein, 1971) and is closely associated with the sites of transmitter release. It generates relatively intense Ca currents during an action potential (Katz & Miledi, 1968, 1970) and is localized to the membrane of the nerve terminal (Katz & Miledi, 1969). This route of Ca entry would promote the phasic release of transmitter by providing for brief periods of time a relative high concentration of Ca ions at the sites of transmitter release (Katz & Miledi, 1968, 1970). However, there may be other routes by which Ca ions could enter the nerve terminal. In squid giant axons the extra influx of Ca associated with electrical activity seems not to be inhibited by Mg (Hodgkin & Keynes, 1957). Thus, it seems possible that during tetanization some Ca ions may enter the nerve terminal by such a Mg-insensitive route, diffuse to the transmitter release sites, and thereby elevate the spontaneous rate of transmitter release. It may be that this route is relatively non-selective and also permits the entry of Mg ions during a tetanus (Baker & Crawford, 1971).

Miledi & Slater (1966) have studied the effects of Ca applied either intracellularly or extracellularly to the presynaptic terminal of the giant

synapse of the squid, and they concluded that the phasic release of transmitter requires Ca only at the external surface of the membrane of the nerve terminal, and not at the internal surface. These findings seem to contradict our suggestion that changes in the intracellular level of Ca may influence the spontaneous rate of transmitter release. However, Miledi & Slater (1966) did not record miniature synaptic potentials which in the giant synapse of the squid are extremely small and difficult to observe (Miledi, 1967).

Many other suggestions have been made to account for the increases in the min.e.p.p. frequencies that have been observed under various experimental conditions. These include the mobilization of transmitter (Eccles, 1957), loss of K (Liley & North, 1953), gain of Na (Birks & Cohen, 1968), and release of Ca from a bound, or sequestered, state within the terminal (Hofmann, 1969; Glagoleva, Liberman & Khashayev, 1970). The displacement of bound Ca from sites within the terminal could be a direct effect of stimulation, or it could result secondarily from the influx of Na or Mg. If displacement of Ca by Na ions were causing the rise in the min. e.p.p. frequency, then the effects of Mg on this frequency might arise secondarily from an effect of Mg on the quantity of Na that entered the nerve terminal during a tetanus. For instance, Mg may have effects similar to Na and other divalent cations and prolong the action potential in the nerve terminal during a tetanus, thereby increasing the quantity of Na that enters with each impulse (Spyropoulos, 1956; Spyropoulos & Brady, 1959; Connelly, 1962). Our results may be compatible with any or all of these ideas, and we cannot decide among them in any conclusive manner. However, the hypothesis that divalent cations accumulate within the nerve endings has the advantage of simplicity and can offer a satisfactory explanation of the effects of the various procedures tested in our experiments. Although this explanation may account for the Ca-independent release of transmitter from tetanized preparations it may not extend to all the nodes of transmitter release that seem to be independent of extracellular Ca. For instance, the marked effects of hypertonic solutions (Blioch *et al.* 1968), black widow spider venom (Longenecker, Hurlbut, Mauro & Clark, 1970) or high concentrations of ethanol (Quastel, Hackett & Cooke, 1971) on the min.e.p.p. frequency may not be related to changes in the internal concentrations of divalent cations.

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