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THE EFFECT OF CALCIUM AND MAGNESIUM ON THE SPONTANEOUS RELEASE OF TRANSMITTER FROM MAMMALIAN MOTOR NERVE ENDINGS

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In both amphibia and mammals the amount of transmitter released at the neuromuscular junction by a nerve impulse varies directly with the calcium concentration and inversely with the magnesium concentration in the surrounding medium (del Castillo & Stark, 1952; del Castillo & Engbaek, 1954; Boyd & Martin, 1956*b*). It might be expected therefore that changes in the concentration of these ions might affect in the same manner the spontaneous release of transmitter that is detected post-synaptically as miniature end-plate potentials (m.e.p.p.s). Previous investigations have shown that in mammalian preparations calcium ions do influence the m.e.p.p. frequency (Boyd & Martin, 1956*a*; Liley, 1956*d*), but in amphibian preparations no consistent effect has been detected (Fatt & Katz, 1952). Magnesium ions have not hitherto been found to change m.e.p.p. frequency in amphibia or mammals (del Castillo & Katz, 1954*a*; Boyd & Martin, 1956*a*).

The results of the present investigation differ from those of previous studies in that both calcium and magnesium have been found to affect m.e.p.p. frequency. This action is, however, exerted on only a fraction of the spontaneous release of transmitter, the remaining fraction being unaffected by the absence of calcium or the presence of magnesium in high concentration.

METHODS

All experiments were performed *in vitro*, with the rat diaphragm–phrenic-nerve preparation (Bülbring, 1946). The animals were male and female albinos of the Wistar strain weighing between 180 and 220 g. The method of dissection, the divided recording chamber and the mounting of the preparation have been previously described (Liley, 1956*a*). The phrenic nerve was immersed in paraffin oil, whereas the strip of diaphragm muscle was irrigated with a solution similar to that described by Liley (1956*a*). Its composition was (mM): NaCl 137·0, KCl 5·0, $CaCl_2$ 2·0, $MgCl_2$ 1·0, NaH_2PO_4 1·0, $NaHCO_3$ 12·0, Glucose 11·0. The experimental solutions resembled this solution except for the omissions or additions of the ion species under test. When the calcium concentration of the fluid was raised above 10 mM it was necessary to omit the NaH_2PO_4 and to reduce the $NaHCO_3$ concentration in order to

keep the calcium in solution. Additions of $MgCl_2$ and $CaCl_2$ were osmotically compensated for by withdrawal of appropriate amounts of NaCl (Heilbrunn, 1952). Compensation was made for added KCl by removal of equimolar amounts of NaCl.

All solutions were bubbled with 95 % O_2 and 5 % CO_2 in a reservoir and then passed, through a drip counter and heating coils in the water-bath, to the muscle chamber. A fine-pointed glass sucker connected to a Venturi pump drew the fluid across the preparation into a collecting flask. At the usual rate of flow (250–500 ml./hr) the temperature in the muscle chamber was 37–38° C. A thermometer in a side compartment of the chamber measured the temperature in all experiments. When higher rates of flow were used to produce rapid changes of the bathing solution, the fluid in the reservoir was heated to about 40° C. Temperatures between 30 and 40° C could be maintained accurately to within 0·5° C by variation of the temperature of the water-bath and of the reservoir of solution.

The pH of the solution, normally 7·3–7·4 at 37° C, was altered in some experiments by variation in the amount of added $NaHCO_3$. In three experiments an alkaline pH was required outside the range of a bicarbonate—CO_2 buffer. Tris (hydroxymethyl) aminomethane was used to give a final pH of 8·6 at 37° C. To keep 10 mM-$CaCl_2$ from precipitating at this pH it was necessary to omit both the $NaHCO_3$ and NaH_2PO_4 from the solution. When pH alterations were made, the final pH of the solution, bubbled with 'Carbogen' at 37° C, was measured with a pH electrometer.

Recording. M.e.p.p.s were recorded with conventional intracellular recording techniques (Ling & Gerard, 1949; Nastuk & Hodgkin, 1950). The micro-electrodes were filled with 3M-KCl and had resistances between 5 and 20 MΩ. The method of pulling and filling these electrodes has been previously described, as have also the micromanipulators (Eccles, Fatt, Landgren & Winsbury, 1954) and electronic recording technique (Brock, Coombs & Eccles, 1952). The indifferent electrode in all experiments was a silver–silver-chloride spiral connected to the solution bathing the preparation by an agar–saline column.

Experimental procedure. The effect of magnesium was measured by comparing the frequency of m.e.p.p.s in the same muscle fibre before, during and after treatment with the magnesium-containing solution. When measuring the effect of calcium the reverse sequence was employed, i.e. solution with added calcium, solution without calcium, solution with added calcium. It was found that the preparation survived for a longer time, and in better condition when kept for two-thirds of the experiment in a calcium-containing medium. If the solution to be washed out contained 10 mM or more of calcium, the sodium salt of ethylene diamine tetracetic acid (EDTA), a chelating agent, was added to the calcium-free solution (in a 1 mM concentration). By this means the removal of calcium was completed in under 15 min, which was about half the time required in the absence of EDTA.

In each preparation the experimental sequence was followed at 8–16 neuromuscular junctions. Often several trials were made at each junction.

M.E.P.P. frequencies were initially measured by photographing successive oscillograph traces and counting m.e.p.p.s from film, usually with some form of magnification. Later a more convenient method of measurement was devised. After amplification the potentials were fed into a dekatron scaler (Isotope Developments Limited) using suitable cut-off bias to exclude base-line noise. The model used had a resolution time better than 3 μsec, and counted random potentials at a frequency of 3000/sec with only a 1 % error. Special care was taken to avoid artifacts and keep base-line noise levels low. Frequencies were normally counted over 100 sec periods, but in solutions in which the potassium concentration was 10 mM or more the m.e.p.p. frequency in the presence of calcium was so high that 40 sec or even 10 sec counting periods gave satisfactory results. It was possible by this method to assess accurately when changes in frequency were fully established. A further advantage was that results were immediately available. In some experiments a photographic record was also made of the m.e.p.p.s. The results were in good agreement with those obtained with the scaler.

RESULTS

During the present investigation it was noticed that m.e.p.p.s could be recorded from muscle fibres bathed in 'calcium-free' solutions for more than 5 hr. In further experiments with calcium-free solutions m.e.p.p. frequencies at individual junctions were stable for up to half an hour, even in the presence of a chelating agent (EDTA) in concentrations up to 5 mM. Even when the calcium-free solutions contained 15 mM-$MgCl_2$, m.e.p.p.s were still recorded at frequencies within the normal range. This continuing release of transmitter, unaffected by wide variations in calcium and magnesium concentration, will be termed the fixed fraction of the spontaneous release. Upon this fixed fraction could be superimposed a fraction whose size is dependent on the concentrations of calcium and magnesium.

Effect of calcium

The effect of calcium-containing solutions on m.e.p.p. frequency was an increase in frequency from the basal level in the absence of calcium. Over the range of calcium concentrations explored (0·20–10 mM), the increase in m.e.p.p. frequency was linearly proportional to the logarithm of the bathing calcium concentration (Fig. 1). The increase in frequency was fully developed within 4–8 min of changing solutions. There was considerable variation in the time which elapsed before any changes in frequency occurred, but thereafter the development was so rapid that it was complete within 2 min.

The effect of calcium on m.e.p.p. frequency was explored in the presence of 0, 1 and 2 mM-$MgCl_2$. It was found that in each case a linear relationship existed between the logarithm of the bathing calcium concentration and the increase in m.e.p.p. The effect of any given calcium concentration was, however, reduced by about 20 % for each 1 mM increase in magnesium concentration (Fig. 1).

Effect of magnesium

In Table 1 the results of nine experiments on one preparation are summarized. They show that a magnesium concentration of 3 mM depressed the frequency of the spontaneous discharge of potentials by about 40 %.

It will be noticed that the m.e.p.p. frequency was not always the same before and after the period of immersion in 3 mM-$MgCl_2$. In order to take account of this a linear change in frequency with time has been assumed, and the average of the final and initial frequencies was compared with the frequency in the test solution (Column 5). A range from 1·0 to 15 mM-$MgCl_2$ was explored in this way in the presence of 2 mM-$CaCl_2$. The results (lowest line of Fig. 2) show that the depression was fully developed at 3 mM.

When the preparations were in solutions containing magnesium at 10 mM or higher concentrations, neuromuscular transmission was blocked and end-plate potentials were recorded in response to stimulation of the

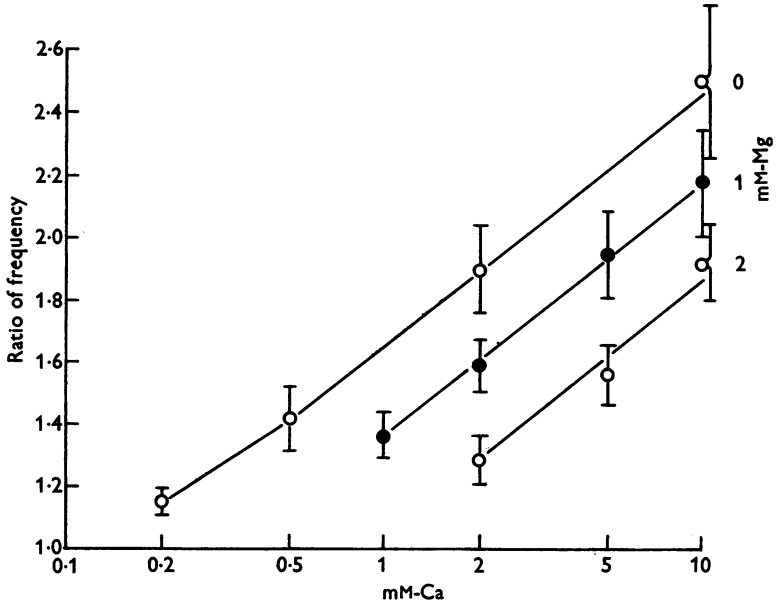

Fig. 1. Influence of calcium concentration upon m.e.p.p. frequency.

Ordinate. Ratio of frequency of m.e.p.p.s in calcium-containing solution to frequency in solution without calcium. Abscissa. Calcium concentration. Upper points obtained in absence of $MgCl_2$, middle in 1 mM-$MgCl_2$, lower in 2 mM-$MgCl_2$. Bars indicate ± 1 S.E. of mean ratio of frequencies.

TABLE 1. Effect of Mg on frequency of m.e.p.p.s

Measurements were made in solution with no added $MgCl_2$ (*A*) and in solution containing 3 mM-$MgCl_2$ (*B*). $CaCl_2$ 2 mM was present throughout. Frequency ratio is calculated from the arithmetic mean of the two *A* values.

Junction	Discharge rates (per second) *A*	*B*	*A*	Frequency ratio *B*/*A*
1	3·68	1·89	2·64	0·599
2	1·04	1·02	2·39	0·591
3	2·13	1·76	2·46	0·764
4	1·78	1·36	1·80	0·760
5	10·94	4·40	2·47	0·655
6	6·38	4·54	7·55	0·651
7	4·32	2·55	4·50	0·577
8	3·25	1·18	2·62	0·402
9	7·88	5·69	7·93	0·719
	Mean and S.E.			0·64 ± 0·04
	Frequency as % of control			64 ± 4

phrenic nerve, even though the depression of m.e.p.p. frequency was then not greater than at lower concentrations of magnesium.

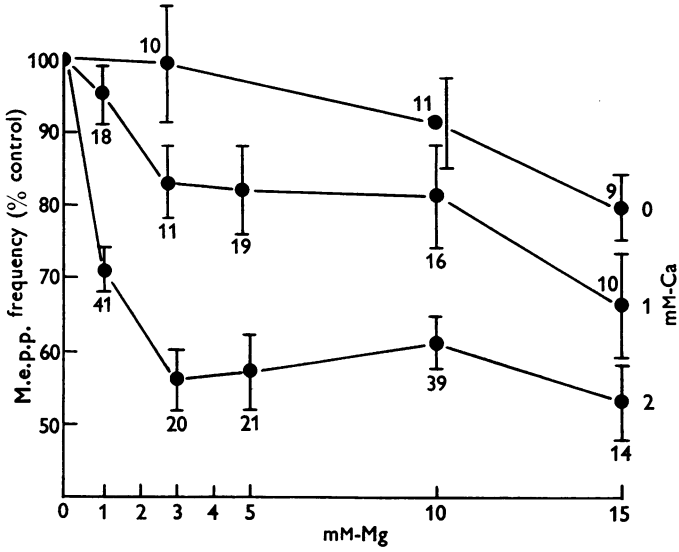

Fig. 2. Influence of magnesium concentration upon m.e.p.p. frequency.

Ordinate. M.e.p.p. frequency as a percentage of frequency in a solution without magnesium. Abscissa. Magnesium concentration. Upper points obtained in absence of $CaCl_2$, middle in 1 mM-$CaCl_2$, lower in 2 mM-$CaCl_2$. Bars indicate ± 1 S.E. of mean depression. Figures under points give number of junctions explored.

The reduction of frequency appeared within 2 min of changing solutions. The effect of temperature on the depression was investigated by repeating the experiments at 31·5–32° C. Over a range of magnesium concentrations from 1 to 10 mM the mean depressions of frequency found were within one standard error of the mean depressions found at 37–37·5° C. Similarly, when the effect of 10 mM-$MgCl_2$ was examined at pH 6·8 (bicarbonate buffer) and at pH 8·6 (Tris buffer), the mean results were almost identical with those at pH 7·4.

Interaction of calcium and magnesium

The results of the present investigation suggest that the action of magnesium was exerted solely by a reduction of the accelerating effect of calcium on m.e.p.p. frequency, as shown in Fig. 1. This would imply that magnesium should be without effect on m.e.p.p. frequency in the absence of calcium. Figure 2 shows that when the calcium concentration of the control and test solutions was reduced to 1 mM the effect of magnesium was greatly reduced over the whole concentration range (1–15 mM). More significantly, repetition of these experiments in the calcium-free

solutions showed that magnesium was indeed now without effect in all but the highest concentrations (uppermost curve of Fig. 2). The magnesium depression of frequency should thus be counteracted either by an absence of

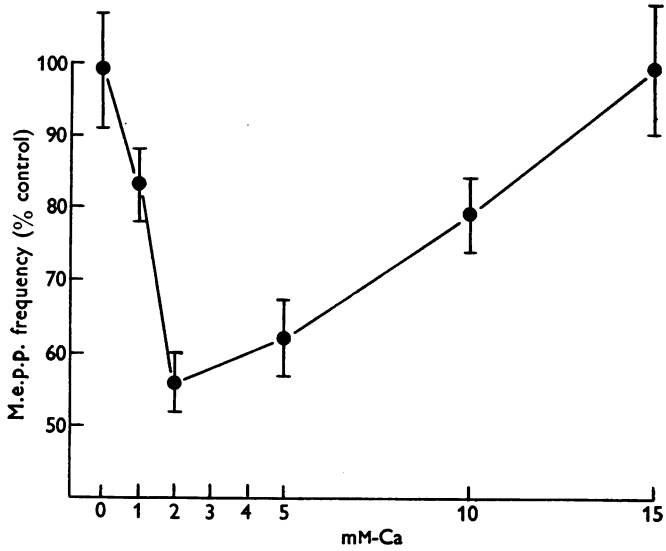

Fig. 3. Influence of calcium concentration on the depression of m.e.p.p. frequency by 3 mM-Mg.

Ordinate. M.e.p.p. frequency as percentage of frequency in a solution without magnesium. Abscissa. Calcium concentration. Bars indicate ± 1 S.E. of mean depression.

calcium or by sufficient excess of calcium to overcome the action of magnesium. This point was tested by exploring the depression of m.e.p.p. frequency by 3 mM-$MgCl_2$ in the presence of 0–15 mM-$CaCl_2$ (Fig. 3). As would be expected from the preceding results, the magnesium depression of frequency was, in fact, at a maximum close to 2 mM calcium.

Effect of depolarization of nerve terminals

The interaction of calcium and magnesium described in the preceding section is of the same type as that suggested by del Castillo & Katz (1956) for the action of these ions on transmitter release by depolarization of nerve terminals. This would suggest that the fraction of the spontaneous transmitter release that is affected by calcium and magnesium might be increased by depolarizing the endings. Raising the potassium concentration of the external medium and thus producing a stable depolarization of nerve terminals (Liley, 1956*c*) was a convenient way of testing this suggestion. The interaction of potassium and magnesium was explored by examining the effect of 3 mM-$MgCl_2$ on the m.e.p.p. frequency in the

presence of 1–20 mM-KCl (Fig. 4). Potassium would be expected to depolarize the nerve terminals appreciably only at concentrations of 10 mM or above (Liley, 1956*c*) and correspondingly there was statistically no

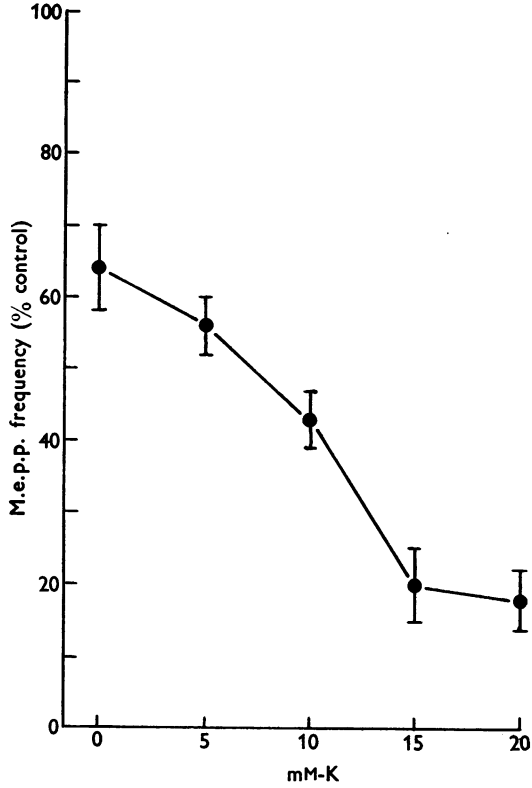

Fig. 4. Influence of potassium concentration on the depression of m.e.p.p. frequency by 3 mM-Mg.

Ordinate. M.e.p.p. frequency as percentage of frequency in solution without magnesium. Abscissa. Potassium concentration. Bars indicate ± 1 S.E. of mean depression.

significant difference between the depressions of frequency in 0 and 5 mM-KCl. The depressions of m.e.p.p. frequency produced by magnesium in the presence of 10, 15 and 20 mM-KCl were progressively much larger than the depression in the presence of 5 mM-KCl, suggesting that the action of magnesium was indeed dependent on the level of depolarization of the nerve terminals.

The accelerating action of calcium on m.e.p.p. frequency on this hypothesis should also be demonstrable in the presence of increased concentrations of potassium. To test this possibility the interaction of calcium and potassium was then explored in a slightly different way. Throughout

a series of experiments the potassium concentration was kept constant at 20 mM, and the effects of 2, 5 and 10 mM-$CaCl_2$ were compared with the effects of a solution containing no calcium but 1 mM EDTA. In six experiments of this type there was no doubt that in the absence of calcium the effect of potassium on m.e.p.p. frequency was greatly reduced. When calcium was introduced the acceleration of frequency was rapid and usually completed within 2–4 min of changing solutions (Table 2). But the mean frequency in 2 mM-$CaCl_2$ (1107 ± 102) was not significantly different from the mean frequency in 5 mM-$CaCl_2$ (1058 ± 46 for 10 measurements) or in 10 mM-$CaCl_2$ (1123 ± 115 for 11 measurements).

TABLE 2. Effect of Ca on frequency of m.e.p.p.s in presence of 20 mM-KCl. Measurements were made in solution with no added $CaCl_2$ but containing 1 mM EDTA (*A*) and in solution containing 2 mM-$CaCl_2$ (*B*). $MgCl_2$ 1 mM was present throughout

	Discharge rates (per second)		
Junction	*A*	*B*	*A*
1	3·60	1426·7	2·54
2	9·39	457·9	—
3	5·70	1206·6	7·5
4	9·09	1308·8	5·7
5	3·99	1205·75	4·61
6	3·31	1275·4	4·1
7	8·11	1069·8	3·45
8	7·12	904·5	2·66
Mean and S.E. in 2 mM-$CaCl_2$ (*B*)		1107 ± 102	—

The possibility was then considered that the increase in m.e.p.p. frequency produced by 20 mM-KCl was so large that an effect of calcium concentration on frequency would be masked. The experiments were therefore repeated with 10 mM-KCl, by both paired and randomized methods, with the same negative result. It is clear that variations in the calcium concentration over the range effective in the presence of 5 mM-KCl (Fig. 1) do not accelerate the m.e.p.p. frequency when the potassium concentration is raised to 10 or 20-mM. As the presence of some calcium is essential for the augmentation of spontaneous activity by potassium (Table 2) a dependence of m.e.p.p. frequency on calcium concentration might, under these circumstances, perhaps be found in a lower range.

Effect of osmotic pressure

Furshpan (1956) has shown that the m.e.p.p. frequency at the frog neuromuscular junction is very sensitive to changes in osmotic pressure and that this sensitivity is not affected by the presence of magnesium. In the present investigation an osmotic pressure difference between control and test solutions of up to 5% affected neither the m.e.p.p. frequency nor the depression of the m.e.p.p. frequency by magnesium. Some experi-

ments were done with solutions made hyperosmotic by adding 10 mM-$MgCl_2$ without any withdrawal of NaCl in compensation ($CaCl_2$ was 2 mM in all solutions). When these solutions were compared with solutions without $MgCl_2$, there was a balance of two effects, the mean result at 37° C (10 mM–0 mM $MgCl_2$) being 0·98 (S.E. 0·11) for 12 measurements, i.e. there was neither an acceleration due to a 9% hyperosmotic solution nor a depression by the magnesium. Five experiments were done with magnesium in 9% hypo-osmotic solution. Here the effects of the two agents were additive, again indicating their independence.

DISCUSSION

The acceleration of m.e.p.p. frequency by calcium (Fig. 1) is of the same order as that described by Boyd & Martin (1956*a*) and Liley (1956*d*), and likewise there was great variability in the magnitude of the acceleration at different junctions with higher calcium concentrations. Magnesium and calcium both penetrate cell membranes slowly (Engbaek, 1952; Hodgkin & Keynes, 1957; Gilbert, 1960). The rapid actions on m.e.p.p. frequency described here are thus probably exerted at surface sites in the nerve membrane. The reciprocal relationships that have been demonstrated for the concentrations of magnesium and calcium on m.e.p.p. frequency (Figs. 1, 2, 3) suggest that both ions act at the same site.

As this action is affected by a potassium-induced depolarization of nerve terminals (Fig. 4) it is likely that the mechanism of transmitter release studied in the present investigation is the same as the voltage-dependent, calcium–magnesium sensitive mechanism proposed by del Castillo & Katz (1956) and Liley (1956*c*) to explain release of transmitter by nerve impulses. Thus, in this preparation the membrane potential must be at a level allowing limited activity of this mechanism. Hyperpolarization of the nerve terminals would then be expected to reduce this activity and thereby to reduce m.e.p.p. frequency, as Liley (1956*c*) has indeed found.

It appears likely that the depressive effect of magnesium on m.e.p.p. frequency described here, was not found by Boyd & Martin (1956*a*) in the cat tenuissimus muscle, because they compared the effect of a solution containing 1·15 mM-$MgCl_2$ with one containing 9·37 mM, both being in the presence of 2·46 mM-$CaCl_2$. The results of the present investigation (Fig. 2) indicate that there would be only a 10% increase in depression in the higher magnesium concentration. Such a small difference would be difficult to detect if only a small number of junctions were examined. In fact, when solutions containing 1 mM-Mg and 10 mM-$MgCl_2$ were compared (in the presence of 2 mM-$CaCl_2$) in the present investigation, no frequency change was found. (Mean ratio of frequencies at sixteen junctions 10 mM–1 mM-$MgCl_2$ was 1·00, S.E. $\pm$ 0·10.)

The absence of any effect of magnesium on m.e.p.p. frequency in the frog (del Castillo & Katz, 1954*a*) cannot be explained in the same way. In these experiments the effect of solutions containing no magnesium and 16·2 mM-$MgCl_2$ were compared. In the present investigation such a change in concentration would have reduced m.e.p.p. frequency at least 45 % (Fig. 2). This difference may indicate that in amphibia the level of the membrane potential of nerve endings is such that activity of the voltage and calcium–magnesium sensitive system is not demonstrable. If this were so, the negligible reduction of m.e.p.p. frequency obtained when nerve terminals are hyperpolarized in frog nerve–muscle preparations would also be explained (del Castillo & Katz, 1954*b*; Kraatz & Trautwein, 1957). A further question raised by this species difference is whether the difference is physiological or merely a reflexion of a greater difficulty of maintaining rat diaphragm preparations in good condition. Similar effects on m.e.p.p. frequency, with calcium at least, have been obtained in the cat tenuissimus preparation (Boyd & Martin, 1956*a*); therefore this criticism cannot be confined to the rat diaphragm. In this connexion it might be expected that, during the course of experiments, as the preparation aged the effect of magnesium would be progressively increased. There was, however, no consistent trend of this kind when results from the first and last two junctions in many experiments were compared. Thus, if there was a depolarization of nerve terminals it did not increase appreciably during the experiments.

The interaction of osmotic pressure and magnesium in the present investigation indicated that these agents affected m.e.p.p. frequency by different mechanisms. In the terms used previously, osmotic pressure increases the fixed fraction, while magnesium affects the voltage and calcium-sensitive fraction. Post-activation potentiation and stretching of the preparation, which increase m.e.p.p. frequency despite concomitant high magnesium concentrations (Liley, 1956*b*; Hutter & Trautwein, 1956), must also act independently of the release mechanism studied in this investigation.

SUMMARY

1. Intracellular recording from neuromuscular junctions in isolated preparations of rat diaphragm has revealed that miniature end-plate-potential frequency is accelerated by calcium and depressed by magnesium. This action appears to be exerted at surface sites in the presynaptic membrane. The action of magnesium is explained as a competition with calcium.

2. Miniature end-plate potentials can still be recorded in the absence of calcium or the presence of high concentrations of magnesium.

3. The mechanism of the fraction of spontaneous transmitter release

which is affected by calcium and magnesium is thought to be the same as that activated by nerve impulses.

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