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ON THE MECHANISM BY WHICH CALCIUM AND MAGNESIUM AFFECT THE RELEASE OF TRANSMITTER BY NERVE IMPULSES

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SUMMARY

1. The relationship between the quantal content of end-plate potentials (e.p.p.s) and the bathing [Ca] and [Mg] was determined at neuromuscular junctions in the rat diaphragm *in vitro*.

2. E.p.p.s were recorded intracellularly from preparations exposed to solutions with [Ca] between 0·05 and 10 mM and [Mg] between 0·1 and 12·5 mM. The quantal content of e.p.p.s was increased by raising the [Ca] over this range and decreased by raising the [Mg]. There appeared to be competition of Mg with Ca at three sites in the nerve terminal membrane.

3. A kinetic scheme based on competition of Ca and Mg at three sites could quantitatively explain the effects of Ca and Mg upon the quantal content of e.p.p.s and also the effects of these ions upon miniature end-plate potential frequency.

INTRODUCTION

The mechanism by which the nerve impulse releases acetylcholine (Ach) from nerve terminals at the neuromuscular junction is not well understood, although the importance of calcium in the process is steadily becoming apparent. Evoked ACh release ceases in nerve-muscle preparations exposed to low Ca concentrations (del Castillo & Stark, 1952), even though conduction of the nerve impulse continues (Katz & Miledi, 1965), while nerve impulses can again release ACh upon extracellular application of Ca just before the nerve impulse reaches the terminal (Katz & Miledi 1967). How Ca acts is not known with certainty. One possibility is that Ca affects release of ACh by combination with receptor sites in the nerve terminal and

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that Mg and Ca compete for these sites (Hubbard, Jones & Landau, 1968). It has also been suggested (Liley, 1956) that the nerve impulse affects transmitter release by accelerating the system responsible for the spontaneous release of ACh measured as miniature end-plate potentials (m.e.p.p.s). An economical hypothesis is that the accelerated system is in fact the Ca receptor system so that the problem of acceleration is reduced to the mechanism of acceleration of the effects of the Ca-receptor combination. This hypothesis was based upon extensive quantitative data on the effects of Ca and Mg on m.e.p.p. frequency at neuromuscular junctions in the rat phrenic nerve-diaphragm preparation (Hubbard *et al.* 1968). The purpose of the present investigation was to provide similar quantitative data on the actions of Ca and Mg upon evoked release in the same preparation so that the hypothesis could be tested by comparison of Ca and Mg actions upon the two forms of release.

METHODS

All experiments were performed *in vitro* using the rat hemidiaphragm with the attached phrenic nerve. The methods were those of a preceding paper (Hubbard *et al.* 1968) except that end-plate potentials (e.p.p.s) were evoked by stimulation of the phrenic nerve which was in oil in one half of the divided recording chamber. Preparations were paralysed either by adding (+)-tubocurarine chloride (Burroughs-Welcome) to the solution or by raising its $MgCl_2$ content to 12·5 mM. The quantal content of e.p.p.s was estimated from the ratio of mean e.p.p. amplitude to mean m.e.p.p. amplitude or from the formula relating quantal content to failures of responses or from the formula relating quantum content to the variance and mean of e.p.p. amplitudes, with the precautions and methods previously described (Hubbard & Løyning, 1966).

Very often regression with time was noticed in the e.p.p. amplitudes, and a computer program (I.B.M. 360) was devised to get the best estimate of the mean quantal content (m) for time zero. In this program the sequence of e.p.p. amplitude readings, after correcting for muscle fibre membrane potential, was divided up into four blocks, the first block consisting of the first fifteen readings, the second block of the first thirty, the third block of the first forty-five and the fourth block of all the readings. For each block an estimate of mean amplitudes was obtained and within each block a regression line of amplitude on time was calculated. This regression line was used in two ways: first to estimate the mean amplitude at time zero, and secondly by using the residual sum of squares after the regression was fitted to obtain an estimate of variance. These two estimates were used according to Martin's (1955) formulae to estimate m at time zero, as well as the standard error of the estimate. For each block, then, a different estimate of m was obtained. To choose the best estimate the sequence of corrected amplitudes was plotted against time, and the longest time over which the regression from the origin was linear was determined. The value of m calculated for the corresponding block was selected.

The time course of e.p.p. amplitude changes following changes in the bathing [Ca] or [Mg] was estimated from records made from the one cell throughout the experiment. In curarized preparations e.p.p.s were elicited at 12·5 or 25 sec intervals.

No osmotic compensation was made for the variation in [Ca] and [Mg] of the bathing solutions in view of the demonstrations by Furshpan (1956) and Gage & Hubbard (1966) that variation of the osmotic pressure of bathing solutions in the range of the present experiments does not affect the quantal content of e.p.p.s.

RESULTS

Time course. The time courses of Ca and Mg actions upon e.p.p. quantal content were the same as the time courses of their actions on m.e.p.p. frequency. In a preceding investigation (Hubbard *et al.* 1968) it was found that the effects of changes of [Ca] upon m.e.p.p. frequency took 15–25 min for completion. As Fig. 1 shows, the amplitude of e.p.p.s evoked at 25 sec intervals changed with a similar time course upon exposure to solutions with a greater (Fig. 1, filled circles) or smaller (Fig. 1, open triangles) [Ca] than that of the control solutions. Figure 1 is representative of five similar

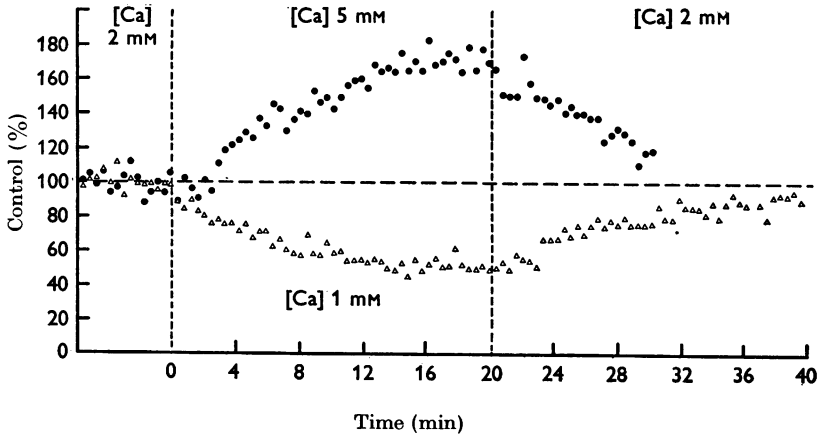


Fig. 1. The time course of the changes in e.p.p. amplitude produced by variation of the bathing [Ca]. Two separate experiments are shown. In both e.p.p.s were recorded at 25 sec intervals in the control solution (2 mM-Ca), then for 20 min in the test solution and again in the control solution. E.p.p. amplitudes are expressed as percentages of the mean amplitude of e.p.p.s in the control solution. In the experiment in which the test solution contained 5 mM-Ca the e.p.p. amplitudes are shown by filled circles and in the experiment in which the test solution contained 1 mM-Ca the amplitudes are denoted by open triangles. The vertical interrupted lines indicate changes of bathing [Ca]. The first indicates the change from the control to the test solution, the second the return from test to control solution.

experiments. These amplitude changes (Fig. 1) presumably reflect changes in quantal content since [Ca] changes of this magnitude, in this preparation, do not change quantal size as estimated from m.e.p.p. amplitude measurements (Hubbard *et al.* 1968).

The effects of Mg upon quantal content also paralleled the effects of this ion upon m.e.p.p. frequency and were similarly complete in a shorter time than the equivalent Ca actions. In three experiments in which the [Mg] of bathing solutions was increased from 1 mM to 3 or 6 mM there was a fall

in the quantal content of e.p.p.s which was complete in 7–9 min and reversed within the same time upon reducing the [Mg] to 1 mM.

Action of Ca. The effects of Ca upon spontaneous release were exhibited in the range 10^{-5}–10^{-2} M (Hubbard *et al.* 1968). The same general range covered the effects of Ca upon ACh release evoked by nerve impulses,

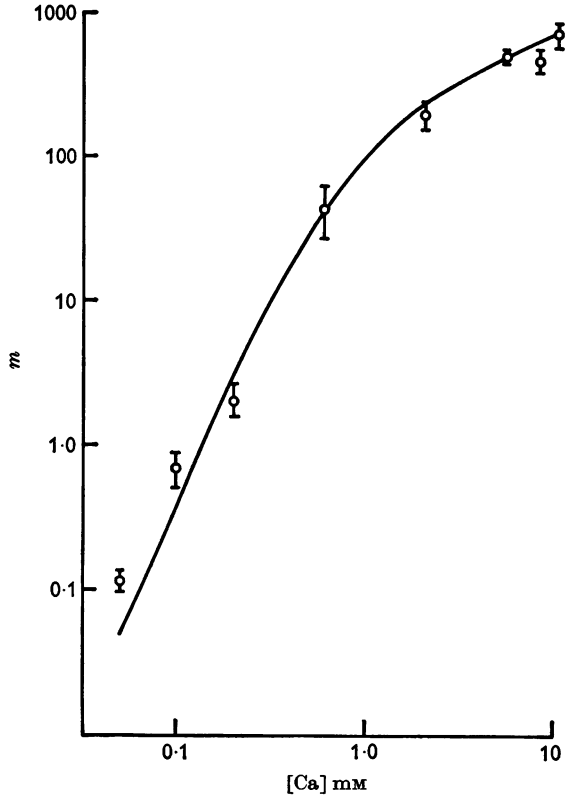


Fig. 2. The relationship between bathing [Ca] and the mean quantal content (m) of e.p.p.s. The $m \pm 1$ S.E. is indicated by the circles and bars. For determination of m, see text. Note logarithmic ordinate and abscissa. The line is computed from equation 2.

although the effects of Ca were much larger. For instance in two experiments it was found that after 2 hr exposure to solutions with a [Ca] of $2{\cdot}4 \times 10^{-5}$ M preparations did not contract upon single or repetitive nerve stimulation. In these preparations also, intracellular recording from junctional regions, identified by the recording of m.e.p.p.s, detected no e.p.p. response upon stimulation. Upon raising the [Ca] of the bathing medium to the normal 2 mM, e.p.p.s were detected upon nerve stimulation and these e.p.p.s rapidly became large enough to generate muscle action potentials,

and thus provoke contraction of the preparation. E.p.p.s could however be recorded in the presence of [Ca] as low as 5×10^{-5} M, although at this concentration responses at individual junctions were intermittent. Weak contraction occurred in response to stimulation at this concentration but was abolished by the addition of (+)-tubocurarine Cl, 5×10^{-8} g/ml. Boyd & Martin (1956) similarly report that most fibres in the cat tenuissimus muscle continued to twitch for an unmentioned time in a solution with no added Ca. As Fig. 2 indicates, m for responses in solutions with [Ca] of 0·05 mM was very low so that it must be assumed either that the threshold for initiation of the muscle action potential had markedly diminished (Costantin, 1967), or, as suggested by Boyd & Martin (1956), that there was a small population of junctions with a larger quantal content.

In agreement with conclusions from previous investigations of the effect of Ca on e.p.p. amplitude and e.p.p. quantal content m was found to increase as [Ca] was increased (del Castillo & Stark, 1952; del Castillo & Katz, 1954; Boyd & Martin, 1956; Liley, 1956; Jenkinson, 1957; Elmqvist & Quastel, 1965). Figure 2 shows m determined over a range of [Ca]s from 5×10^{-5} to 1×10^{-2} M. Each estimate of m represents the arithmetic mean (± 1 S.E.) of quantal contents estimated (see Methods) from 6 to 14 junctions in preparations bathed at that particular [Ca]. The data have been plotted on logarithmic co-ordinates because of the wide ranges involved, and also to allow comparison with the form of results from previous related investigations.

In low [Ca]s the relationship between [Ca] and m (Fig. 2) was almost linear, with a gradient of approximately 2·6. This compares with a gradient of 4 for a similar comparison of e.p.p. amplitude and [Ca] at the frog neuromuscular junction (Dodge & Rahamimoff, 1967). However, the data of Jenkinson (1957) using the same frog preparation yields a gradient of 3. In higher [Ca]s, the relationship deviates from linearity and m appears to be approaching a maximum. A similar result was found for the relationship between e.p.p. amplitude and [Ca] reported by Jenkinson (1957).

Action of Mg. The effect upon m of a 12·5-fold increase in [Mg] (from 1×10^{-3} to $12{\cdot}5 \times 10^{-3}$ M), and of a 10-fold decrease in [Mg] (from 1×10^{-3} to 1×10^{-4} M) was investigated in a range of [Ca]s. As for Fig. 2, each estimate of m represents the arithmetic mean (± 1 S.E.) of quantal contents estimated (see Methods) from 6 to 14 junctions in preparations bathed at that particular [Ca]. As Fig. 3*A* shows, m fell as [Mg] increased, in agreement with the conclusions of previous investigations (del Castillo & Engbaek, 1954; del Castillo & Katz, 1954; Boyd & Martin, 1956; Jenkinson, 1957).

The relative decrease in m produced by increasing [Mg] was greater in

low [Ca]. This would be expected from the evidence of Jenkinson (1957) that Ca and Mg compete in their effects upon quantal content. The data of Fig. 3*A* have been replotted in Fig. 3*B* to show the relationship between the reciprocal of *m* (using a logarithmic scale for convenience) and the reciprocal of [Ca], at various [Mg]. Extrapolation of all three curves to a common intercept on the ordinate would indicate competitive interaction between Ca and Mg. As can be seen from Fig. 3*B*, the data are suggestive

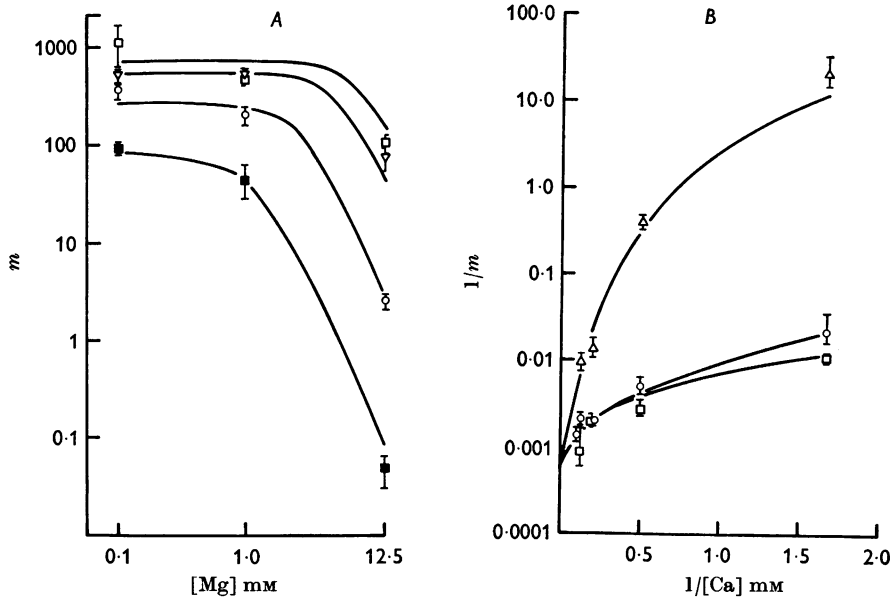


Fig. 3. The interaction of Ca and Mg. *A*. Quantal contents ($m \pm 1$ S.E.) of e.p.p.s in three [Mg]s and 0·6 (filled squares), 2 (circles), 5 (triangles) and 8 (open squares) mM-Ca. For determination of *m*, see text. Note logarithmic scales. *B*. Competition between Ca and Mg. Points indicate $m \pm 1$ S.E. Note reciprocal scales. Open triangles 0·1 mM-Mg, open circles 1 mM-Mg and open squares 12·5 mM-Mg. The lines in *A* and *B* are computed from equation 2.

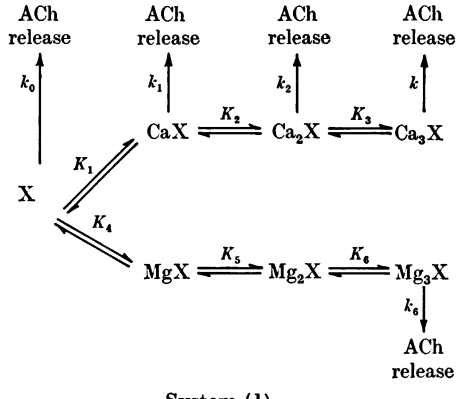
of such a relationship, although no firm conclusion can be drawn because of the standard errors of the results and the curved form of the plot.

Comparison of the effects of Ca and Mg upon spontaneous and evoked ACh release. In an investigation of the effects of Ca and Mg on spontaneous ACh release (Hubbard *et al.* 1968) it was concluded that Ca and Mg may act on spontaneous transmitter release by competing at two or more sites on a Ca/Mg binding nerve terminal receptor molecule active in the process leading to transmitter release, so modifying its activity. Dodge & Rahamimoff (1967) have recently proposed, from the gradient of a log–log plot of e.p.p. amplitude as a function of [Ca] that at the frog neuromuscular

junction a nerve terminal receptor bearing four Ca molecules may be involved in evoked transmitter release. In a similar manner (Fig. 2) it appears likely that a molecule binding three Ca molecules is involved in evoked ACh release at the mammalian neuromuscular junction.

Further evidence suggesting the binding of three Ca molecules was obtained by plotting the reciprocal of m as a function of [Mg] on logarithmic co-ordinates. It can be shown that the gradient of such a plot, for quantal contents determined in high [Mg]s at low [Ca], is an indication of the number of sites Mg may occupy on the proposed receptor molecule. When the data of Fig. 3*A* were plotted in this form the gradient for high [Mg]s at low [Ca]s was 2·7.

As spontaneous release appears to involve activity of a receptor with two or more sites at which Ca and Mg compete (Hubbard *et al.* 1968) and as evoked release appears to involve activity of a receptor with at least three sites, at which Ca and Mg compete, the hypothesis that Ca and Mg affect a receptor system common to, and participating in, both spontaneous and evoked ACh release can be maintained. The ability of such a hypothesis to explain quantitatively the actions of Ca and Mg in both forms of transmitter release was therefore tested on the assumption of system (1).



System (1)

*K*s = dissociation constants, *k*s = rate constants, $k = k_3$ for spontaneous ACh release, $k = k_3'$ for evoked ACh release, where $k_3' > k_3$.

This system is of the form previously postulated to explain spontaneous release (Hubbard *et al.* 1968) but with three instead of two Ca/Mg receptor sites. The change in the system which is proposed to explain the increased release rates provoked by nerve impulses and the lack of excitation of evoked release by Mg is an increase in the rate constant of the Ca_3X species (k_3). This change was the simplest which could quantitatively explain the

relationship between [Ca] and [Mg] and release found for evoked release (Figs. 2, 3) and spontaneous release (Hubbard *et al.* 1968).

Alternative means of producing an acceleration of such a system, and reasons for rejection were: (1) An increased local [Ca]. This is unlikely, for in spontaneous release the receptor appears to be almost saturated by a [Ca] of 1×10^{-2} M (Hubbard *et al.* 1968), when release rates are of the order of 5–10 quanta/sec only. (2) Increased total receptor, X_T. This would predict that the ratio, evoked release rate/spontaneous release rate, be constant for all [Ca]. In fact a sigmoidal relationship is obtained. (3) A change in dissociation constants to produce greatly increased quantities of an active Ca-bound species. This is unlikely because in spontaneous release maximal formation of the Ca_3X complex produces frequencies of 5–10 quanta/sec, while predominant formation of Ca_2X or CaX complexes would predict gradients of 2 or less for the results as plotted in Fig. 2. (4) Increases in rate constants of the various species could occur. However the gradient of the relationship between [Ca] and quantal content is approximately 2·6 in low [Ca] (Fig. 2) suggesting the importance of a receptor species bearing more than two Ca molecules. Hence a change in the rate constant of Ca_3X alone appears the simplest change to consider.

The following rate equations (equations (1) and (2)) for the system being considered were derived in the manner previously described (Hubbard *et al.* 1968). With use of a curve-fitting computer program constants were determined and the ability of this system to explain the experimental data for spontaneous and evoked release assessed.

Quantal release rate

$$= \frac{V_m\left(\frac{k_0}{k} + \frac{k_1}{kK_1}[\mathrm{Ca}] + \frac{k_2}{kK_1K_2}[\mathrm{Ca}]^2 + \frac{1}{K_1K_2K_3}[\mathrm{Ca}]^3 + \frac{k_6}{kK_4K_5K_6}[\mathrm{Mg}]^3\right)}{\left(1 + \frac{[\mathrm{Ca}]}{K_1} + \frac{[\mathrm{Ca}]^2}{K_1K_2} + \frac{[\mathrm{Ca}]^3}{K_1K_2K_3} + \frac{[\mathrm{Mg}]}{K_4} + \frac{[\mathrm{Mg}]^2}{K_4K_5} + \frac{[\mathrm{Mg}]^3}{K_4K_5K_6}\right)}, \quad (1)$$

where V_m = quantal release rate when [Ca] is saturating.

The acceleration of release following the action potential is clearly gross; thus for evoked release the activity of complexes other than the Ca_3X species is insignificant on the system being considered. The rate equation (1) may therefore be simplified for evoked release, to

Quantal release rate

$$= \frac{V_m[\mathrm{Ca}]^3}{K_1K_2K_3\left(1 + \frac{[\mathrm{Ca}]}{K_1} + \frac{[\mathrm{Ca}]^2}{K_1K_2} + \frac{[\mathrm{Ca}]^3}{K_1K_2K_3} + \frac{[\mathrm{Mg}]}{K_4} + \frac{[\mathrm{Mg}]^2}{K_4K_5} + \frac{[\mathrm{Mg}]^3}{K_4K_5K_6}\right)}. \quad (2)$$

As this equation does not contain rate constants, units different from those used to measure spontaneous release may be used to estimate evoked release, provided of course that the V_m for evoked release is also estimated in the same new units. Thus using e.p.p. quantal content as the parameter of the rate of evoked release, quantal release rate in equation (2) may be replaced by quantal content.

The following constants were used in equations (1) and (2) to provide theoretical curves based on the system being considered:

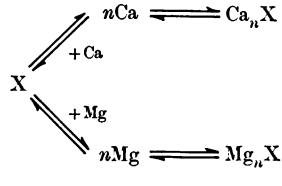
K_1	7×10^{-3} M	k_0	$= 6{\cdot}21 \times 10^{-2} k_3$
K_2	5×10^{-6} M	k_1	$= 2{\cdot}17 \times k_3$
K_3	1×10^{-2} M	k_2	$= 1{\cdot}09 \times 10^{-1} k_3$
K_4	$3{\cdot}5 \times 10^{2}$ M	k_6	$= 2{\cdot}59 \times 10^{-1} k_3$
K_5	$5{\cdot}56 \times 10^{-6}$ M	V_m	for spontaneous release 9·2 quanta/sec.
K_6	$9{\cdot}33 \times 10^{-4}$ M	V_m	for evoked release $1{\cdot}6 \times 10^{3}$ quanta/nerve impulse

A simple increase in the rate constant for the Ca_3X complex was found to give a satisfactory quantitative explanation of the effects and interactions of Ca and Mg on e.p.p. quantal content. The line in Fig. 2 shows that the theoretical relationship between [Ca] and e.p.p. quantal content at [Mg] 1×10^{-3} M approximates the experimental data well. Similarly, as Figs. 3*A* and 3*B* show, the theoretical lines for the relationship between e.p.p. quantal content and [Mg] in a range of [Ca]s provide satisfactory approximations to the observed data.

The scheme was further tested by determining m.e.p.p. frequencies expected for the [Ca] and [Mg] explored by Hubbard *et al.* (1968). Figures 4*A* and 4*B* show the previously reported spontaneous quantal rates determined over a range of [Ca]s and [Mg]s respectively (Hubbard *et al.* 1968), and the calculated curves for system (1).

The reported effects of [Ca] on spontaneous release at various [Mg] (Hubbard *et al.* 1968) were also satisfactorily explained in form by system (1), although the m.e.p.p. frequencies recorded particularly for lower [Mg]s were lower than those predicted. This was expected, for the data were obtained from preparations exposed to solutions of lower osmolarity than the data of Figs. 2 and 3*A* and 3*B*, to which the fittings were made.

A mathematically simpler scheme was considered of the form



where no account was taken of the possibility of formation of intermediate Ca or Mg complexes (Dodge & Rahahimoff, 1967). However such a system did not quantitatively explain the observed relationship between Ca and Mg concentration and m.e.p.p. frequency (Hubbard *et al.* 1968) or the relationship between Ca and Mg concentration and e.p.p. quantal content (Figs. 2, 3). Furthermore, there is no reason to suppose that complexes bearing less than the maximum number of Ca or Mg molecules cannot be formed.

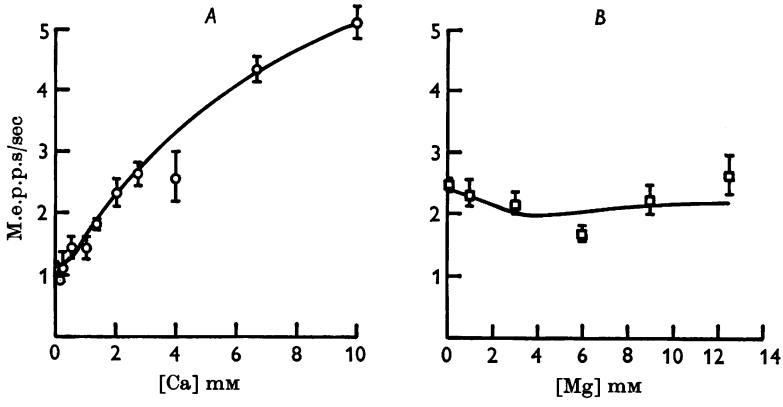


Fig. 4. The relationship between [Ca] and m.e.p.p. frequency (*A*) and [Mg] and m.e.p.p. frequency (*B*) from Hubbard *et al.* 1968. Note the linear scales and the lines, computed from equation (1).

In the scheme proposed, no change in the activity of any Ca or Mg complex other than the Ca_3X complex has been proposed. Our results do not exclude such changes but the most satisfactory theoretical fits to the experimental data required dominant activity from a complex bearing three Ca molecules.

An estimate of the relative increase in activity of the Ca_3X species in such a system may be made by assuming a conversion factor (A) allowing expression of quantal content as a release rate in the units used for measuring spontaneous release (quanta/sec). That is m, mean quantal content $= mA$ quanta/sec. Thus, for evoked release, $V_m = 1600\,A$ quanta/sec. Now $V_m = X_T k$ (where $k = k_3$, $V_m = 9{\cdot}2$ quanta/sec, for spontaneous release; and $k = k_3'$, $V_m = 1600\,A$ quanta/sec, for evoked release).

Thus

$$k_3' = \frac{1600\,A\,k_3}{9{\cdot}2}. \tag{3}$$

It is probable that the release of quanta by a nerve impulse is complete within less than a msec so that in mammalian preparations A is of the order of 10^3–10^4. From (3), this would indicate an approximately 10^6-fold increase in the activity of Ca_3X.

DISCUSSION

The results reported here, taken in conjunction with the results of a previous investigation (Hubbard *et al.* 1968), provide quantitative data on spontaneous and evoked quantal release rates in the one preparation, over a wide range of [Ca] and [Mg] concentrations. Consideration of these results, in the light of the view that spontaneous and evoked ACh release occur through an essentially common release system, has suggested a common kinetic system on which Ca and Mg interact. This system provides an explanation of the effects of Ca and Mg upon spontaneous and evoked release, and suggests a mechanism for the acceleration of quantal release that results in an e.p.p. While there is good evidence for a Ca receptor system in the nerve terminal membrane (Hubbard, 1961; Miledi & Slater 1966; Hubbard *et al.* 1968) the proposed acceleration of the effects of Ca receptor combination by a nerve impulse is at present only speculative. In its favour it must be said that, given the proposed three Ca acceptor system, which gives a good quantitative fit (eqn. (2)) to the effect of a wide range of Ca and Mg concentrations upon spontaneous release (Fig. 4), by a simple increase in activity of the proposed multi-Ca receptor species a quantitative explanation of the relationship between Ca, Mg and evoked release is also provided (Figs. 2, 3).

Two possible mechanisms for the increased activity of the Ca-receptor complex may be suggested. The first of these is entry of the complex into nerve terminals. Entry of Ca into nerve terminals as a result of terminal depolarization by the nerve impulse was suggested by Hodgkin & Keynes (1957) and indeed there is evidence from squid axon, crab nerve and skeletal muscle that Ca entry into these tissues is accelerated as a result of membrane depolarization (Flückiger & Keynes, 1955; Keynes & Lewis, 1956; Hodgkin & Keynes, 1957; Bianchi & Shanes, 1959). The work of Douglas and his colleagues upon adrenal chromaffin cells and the neurohypophysis indicates that hormone secretion is induced by Ca entry following depolarization of the secreting element (Douglas & Poisner, 1964*a*, *b*, Douglas, Kanno & Sampson, 1967). Similarly the experiments of Katz & Miledi (1966) upon transmitter release from frog motor nerve terminals have been explained by the entry of a positively charged Ca species into the nerve terminals during terminal depolarization.

A second possibility, which is of some appeal in view of the essentially allosteric effect of Ca upon transmitter release which we have proposed, is that the action potential produces a conformational change in the nerve terminal membrane as suggested by Bass & Moore (1966) and this change results in a greatly increased activity of the proposed Ca complex.

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