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The inhibitory effect of manganese on transmitter release at the neuromuscular junction of the toad

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Summary

1. Manganese, at low concentrations (0·5–2·0 mM), blocks neuromuscular transmission in toads. Endplate potentials (e.p.ps) are reduced in amplitude but the amplitude of miniature endplate potentials (m.e.p.ps) is, if anything, increased.

2. The release of transmitter by an action potential is reduced in solutions containing Mn, but is still well described by the Poisson equation.

3. Log–log plots of e.p.p. quantal content (m) against [Ca] have a mean gradient of 3·73, and a model based on the co-operative action of four calcium ions in excitation–secretion coupling, and competitive inhibition by Mn, was constructed. The model, with its exponent of 4, is shown in fact to predict gradients of less than four for log–log plots of m against [Ca].

4. The assumption of competitive inhibition by Mn was supported by modified Lineweaver–Burk plots of $m^{-\frac{1}{4}}$ against 1/[Ca]. The mean dissociation constants for Ca and Mn were 1·3 mM and 0·15 mM respectively. From the model, an upper estimate of the density of calcium 'receptor sites' of 30 per square micron of nerve terminal was obtained.

5. In contrast to its inhibitory effect on evoked release, Mn increases the spontaneous release of transmitter. It is suggested that Mn may increase m.e.p.p. frequency by releasing Ca from an intracellular store.

Introduction

It is well established that calcium ions are essential for normal synaptic transmission at 'chemical' synapses. Recent evidence (Katz & Miledi, 1965, 1967a, b; 1969a, 1970) supports the hypothesis that calcium moves across the presynaptic membrane in response to membrane depolarization. Voltage-dependent increases in calcium permeability have been shown to be inhibited by manganese ions in squid axon (Baker, Hodgkin & Ridgway, 1971), barnacle muscle (Hagiwara & Nakajima, 1966), smooth muscle of guinea-pig taenia coli (Hashimoto & Holman, 1967; Bülbring & Tomita, 1969) and in rat and rabbit myometrium (Coraboeuf & Vassort, 1967). Manganese ions are also known to inhibit synaptic transmission (Miledi, 1966; Katz & Miledi, 1969b) and have been shown to reduce transmitter release from sympathetic nerve endings in cats (Kirpekar, Dixon & Pratt, 1970).

The object of this work was to investigate the effect of manganese ions on neuromuscular transmission. At the neuromuscular junction transmitter release can be measured in terms of the number of quanta of transmitter released by an action

potential. It seemed very likely that manganese ions, like magnesium ions (Del Castillo & Engbaek, 1954; Jenkinson, 1957; Dodge & Rahamimoff, 1967) might inhibit secretion of transmitter by 'competing' with calcium ions so that the influx of calcium in response to membrane depolarization is reduced. It was found that manganese did inhibit the evoked release of acetylcholine and the results indicate competition between calcium and manganese ions in excitation–secretion coupling. Some of these results have been published in abstract previously (Balnave & Gage, 1971) and a recent brief report (Meiri & Rahamimoff, 1972) describes similar results.

Methods

The influence of manganese on transmitter release was investigated in the isolated sciatic–sartorius preparation from the Queensland cane toad *Bufo marinus*. Miniature endplate potentials (m.e.p.ps) and endplate potentials (e.p.ps) were recorded with conventional, intracellular microelectrode recording techniques. The sciatic nerve was stimulated at 0·4 Hz and 100–250 endplate potentials (e.p.ps) were averaged using a digital computer on line (Lab 8 system, Digital Equipment Corporation, Mass.). An average waveform, with 95% confidence limits (Fig. 1) and an indication of any regression of e.p.ps during the analysis, were displayed on an oscilloscope and photographed. Where regression occurred (due, for example, to a change in membrane potential) the results were not used.

The normal toad saline had the following composition (mM): NaCl 115, KCl 2·5, $CaCl_2$ 1·8, $NaHCO_3$ 6·0. When bubbled with 95% oxygen and 5% CO_2 the pH at 20° C was between 7·2 and 7·3. HEPES buffer (*N*-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid, Calbiochem) (Good, Winget, Winter, Connoly, Izawa & Singh, 1966), at a concentration of 0·3 mM was substituted for bicarbonate in later experiments to avoid the formation of insoluble manganese carbonate which sometimes occurred in solutions. Solutions containing HEPES buffer were titrated to pH 7·2 with NaOH before addition of manganese chloride (B.D.H.). No osmotic or ionic compensation was made for the addition of Mn (2 mM or less) or for small changes in calcium concentration. Since most procedures involved following the effect of various concentrations of these two ions on transmitter release at the same endplate, a rapid flow-through system was used and preparations were left for 20–30 min to equilibrate with each new solution. A bracketing procedure was used: after a series of solutions had been used, one of the first solutions was retested, and if the average e.p.p. was significantly different from that recorded during the first exposure, the series of measurements was not used. A thermistor probe in the muscle bath was used to monitor the temperature and this was maintained constant (19–21° C) throughout each experiment by means of a constant-temperature water jacket around the bath.

Results

Endplate potentials

In solutions containing 1–2 mM manganese (with the normal concentration of Ca), neuromuscular transmission was blocked and muscles stopped twitching in response to nerve stimulation. When a muscle had stopped twitching, a microelectrode was inserted in an endplate region to record the endplate potentials. These were found

to be reduced in amplitude. Furthermore, when 2 mM manganese was used, there was often no postsynaptic response to a presynaptic stimulus and when endplate potentials did occur, their amplitudes fluctuated over a wide range.

Because of the wide variation in e.p.p. amplitude from endplate to endplate, the relationship between e.p.p. amplitude, quantal content, and Mn concentration was investigated by exposing one endplate to a series of concentrations of Mn, allowing 20–30 min for equilibration with each solution. If recording was started before that time, regression of e.p.ps was sometimes observed.

The effect of [Mn] on the mean amplitude of e.p.ps is illustrated in Figure 1. Solutions contained 1·8 mM Ca^{++} and 2 mg/litre (+)-tubocurarine chloride (Drug Houses of Australia) and the e.p.ps were averaged by computer. Tubocurarine was used when e.p.p. amplitudes were expected to exceed 5 mV, when non-linear summation of quantal responses might be expected (Martin, 1955). The thickness of the lines in Fig. 1 (or the two outer lines where they are distinct) shows the 95% confidence limits of the mean. E.p.p. amplitude is plotted against Mn concentration

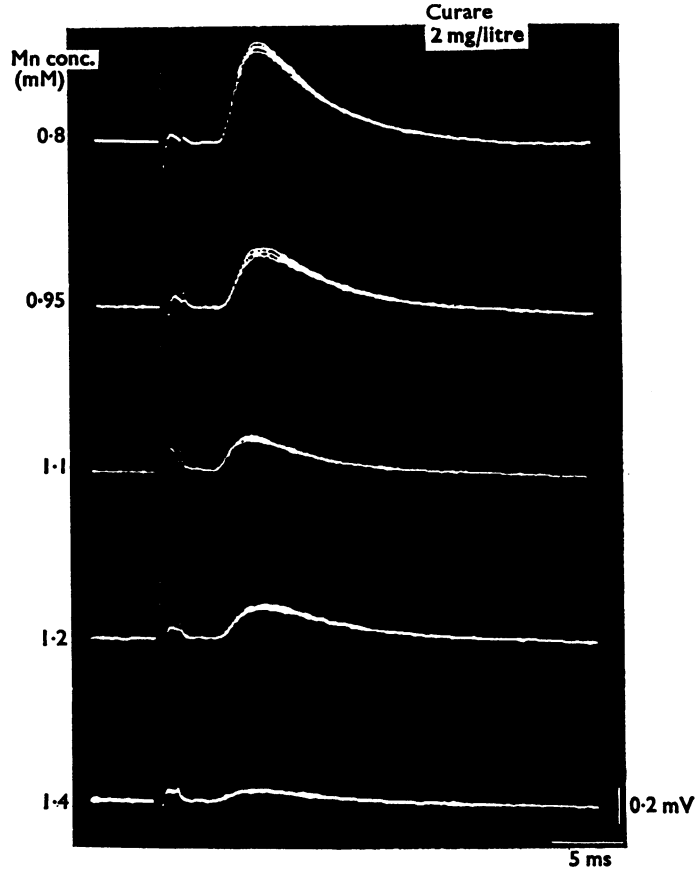

FIG. 1. Averaged endplate potentials in solutions containing 1·8 mM Ca, tubocurarine (curare) 2 mg/litre, and the Mn concentrations shown on the left. The average waveform is accompanied by lines, above and below, which show the 95% confidence limits. These lines are in general so close that they have merged with the average, forming a thicker trace. Temp. 20° C.

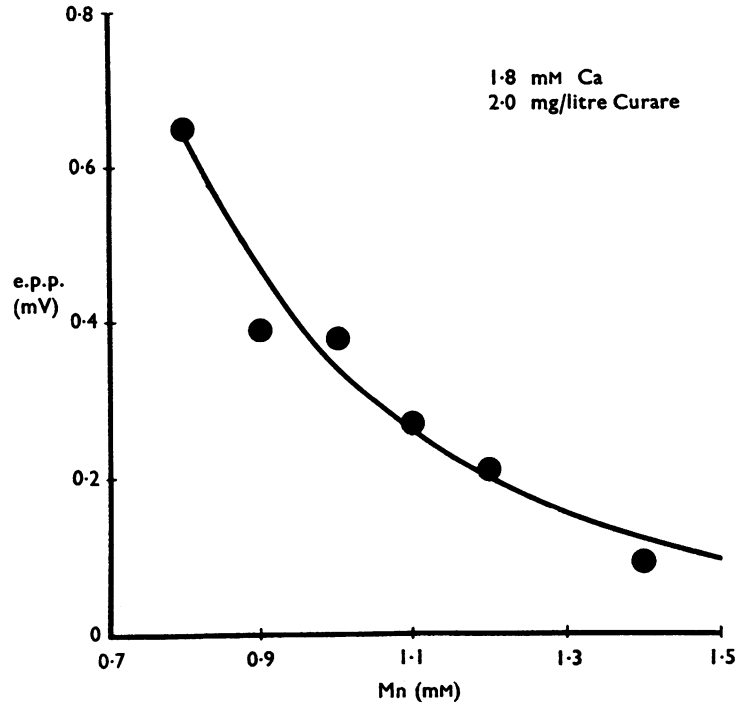

FIG. 2. The relationship between average endplate potential amplitude and manganese concentration in a solution containing 1·8 mM Ca and tubocurarine 2 mg/litre. The line drawn through the points was derived from eqn. (3), with dissociation constants (K_1 and K_2) of 1·3 and 0·15 mM, and a proportionality constant chosen to superimpose observed and calculated e.p.ps at [Mn]=0·8 mM.

in Figure 2. The relationship is clearly non-linear and rather steep. A less than two-fold increase in Mn concentration reduced the e.p.p. amplitude to about one-seventh.

Although Mn markedly reduced the amplitude of e.p.ps it did not reduce the sensitivity of the postsynaptic membrane to the transmitter. In fact, in solutions containing more than 1 mM manganese there was a delayed increase in the amplitude of miniature endplate potentials (m.e.p.ps). The increase in amplitude depended on the concentration of Mn. In solutions containing 2 mM Mn, m.e.p.p. amplitude increased by up to 60%. One of these experiments is illustrated in Figure 3A. At lower Mn concentrations, smaller increases in m.e.p.p. amplitude were seen. These observations excluded the possibility that the reduction in e.p.p. amplitude caused by Mn could be caused postsynaptically but made it necessary to measure quantal content of e.p.ps rather than e.p.p. amplitude as a measurement of transmitter release. The cause of the increase in m.e.p.p. amplitude was not investigated.

If m.e.p.ps cannot be recorded (e.g. in solutions containing tubocurarine) it is generally possible to determine the average quantal content of e.p.ps from the variance in amplitude of e.p.ps or from the number of failures of response to an action potential (Del Castillo & Katz, 1954b ; see Martin, 1966, for review). These methods are based on the observation that the distribution of the release of quanta conforms to a Poisson equation. Therefore the applicability of the Poisson equation was tested for e.p.ps in Mn solutions.

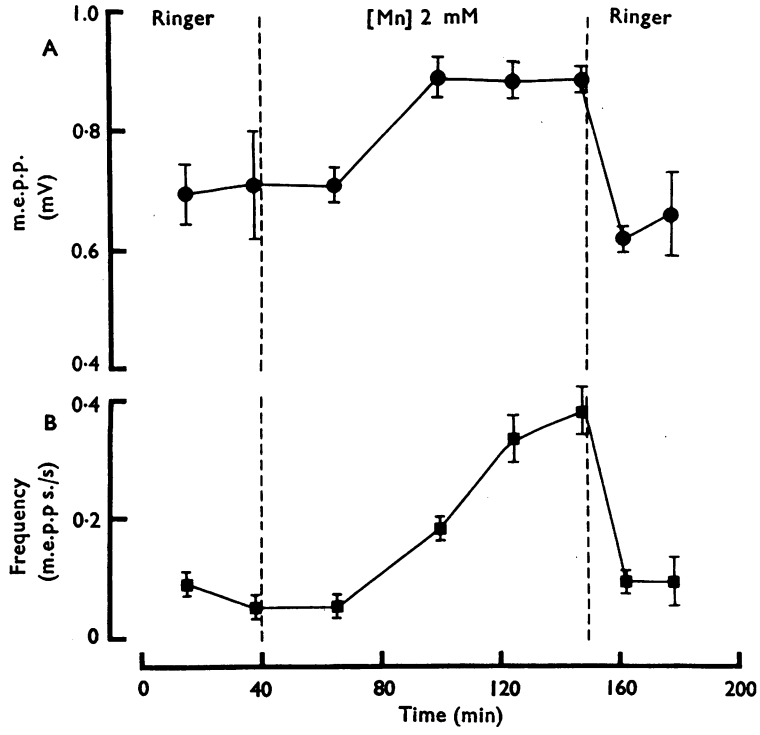

FIG. 3. The effect of 2 mM manganese on miniature endplate potentials. A, Amplitude of m.e.p.ps. B, Frequency of m.e.p.ps. The calcium concentration was 1·8 mM. The vertical bars show ±1 S.E.M.

TABLE 1. *Poisson distribution of quantal content in solutions containing manganese*

	Preparation I				Preparation II					
Number of quanta:	0	1	2	>2	0	1	2	3	4	>4
Observed	225	60	10	1	101	93	49	11	0	0
Predicted	223	63	9	1	103	93	42	12	3	1
χ^2 level		58%					25%			
Manganese		1·4 mM					2·0 mM			
Number of trials		296					254			
e.p.p./m.e.p.p.		0·28					0·90			
ln (stimuli/failures)		0·27					0·92			
$1/C.V.^2$		0·27					1·11			

Poisson release in Mn

It was found that the conformity to a Poisson distribution, where tested in muscles equilibrated in solutions containing 1·8 mM Ca and 1·4 mM Mn (preparation I), and 1·8 mM Ca and 2 mM Mn (preparation II), was good. The results of two such tests are shown in Table 1. In preparation I, for example, 296 e.p.ps and 88 m.e.p.ps were recorded. On the assumption that the amplitude of the quantal responses contributing to e.p.ps were the same as the amplitudes of m.e.p.ps, the mean number of quanta in an e.p.p. (the 'quantal content') was calculated by dividing mean e.p.p. by mean m.e.p.p. amplitude. The quantal content obtained in this way in preparation I was 0·28 and this mean value was used in the Poisson equation to predict the number of failures, unit responses, double responses etc.

These predicted numbers are shown to the nearest integer in the Table. A histogram of e.p.p. amplitudes was divided into groups based on the amplitude of m.e.p.ps, and the observed number of failures, unitary responses etc. is shown in the Table. The agreement between predicted and observed responses is clearly very good, both in this preparation and in preparation II (Table 1). The chi-square level was 58% in preparation I and 25% in preparation II.

When quantal content (m) was calculated from the relationships, $m=\ln$ (number of stimuli/number of failures), or $m=(1/\text{co-efficient of variation of e.p.ps})^2$, which depend on the Poisson equation, the agreement with the values of quantal content derived from the ratio of e.p.p. to m.e.p.p. amplitude was very good (Table 1). Whenever possible, m.e.p.p. amplitude was used to calculate quantal contents in different manganese solutions. However, it was often necessary to use the other two methods.

The relationship between quantal content and [Ca]

The aim of the experiments was to investigate whether Mn inhibits release of transmitter by competing with Ca. The relationship between transmitter release and [Ca] has been found not to be linear and apparently conforms to a fourth (Dodge & Rahamimoff, 1967) or third (Hubbard, Jones & Landau, 1968b) power relationship. It was necessary to define the relationship at the toad neuromuscular junction before testing for competition between the two ions. An attempt was made to do this by plotting the logarithm of quantal content against the logarithm of [Ca] (in solutions containing 0·5 to 1·25 mM Mn) and measuring the gradient of the line through the points. The gradients obtained in fourteen experiments are shown in Table 2. The mean gradient was 3·73 (S.E.M.=0·14), which is in good agreement with the mean value of 3·78 obtained by Dodge & Rahamimoff (1967).

TABLE 2. *Gradients of lines relating log quantal content to log [Ca]*

Junction	Mn (mM)	Ca range (mM)	Gradient of log-log graph
1	1·25	0·75–1·00	4·7
2	1·25	1·00–1·50	4·2
3	1·00	0·75–1·25	3·6
4	1·00	0·75–1·25	4·0
5	1·00	0·37–0·87	3·4
6	1·00	1·00–1·25	4·4
7	1·00	1·00–2·00	3·2
8	1·00	1·00–1·50	3·3
9	0·75	0·75–1·12	4·3
10	0·75	0·90–1·20	3·7
11	0·50	0·25–0·62	3·4
12	0·50	0·30–0·75	3·3
13	0·50	0·50–1·00	3·2
14	0·50	0·50–1·00	3·3
			mean=3·73
			S.E.=0·14

For the purpose of exploring the influence of Ca and Mn on the transmitter release process, and their possible interaction, the following simple 'working hypothesis' or model was adopted. It is assumed that during, or immediately following, an action potential calcium ions can bind to a site or acceptor, X, forming a complex CaX (cf. Del Castillo & Katz, 1954a), and that Mn can also bind to X forming MnX. If the concentration of 'receptor sites' per nerve terminal is small compared

with the concentrations of Ca ([Ca]) and Mn ([Mn]), the following equation, based on equilibrium equations, can be written (cf. Gage & Quastel, 1966):

$$[CaX] = \frac{[X_T] \cdot \frac{[Ca]}{K_1}}{1 + \frac{[Ca]}{K_1} + \frac{[Mn]}{K_2}} \tag{1}$$

where K_1 and K_2 are dissociation constants for CaX and MnX respectively, and $[X_T]$ is the total concentration of sites. The probability of Ca binding to X, p(Ca), $\left(= \frac{[CaX]}{[X_T]}\right)$ is equal to $\frac{[Ca]/K_1}{1 + [Ca]/K_1 + [Mn]/K_2}$. The probability of n such sites co-operatively occurring, p(n.Ca), ($= [p(Ca)]^n$) is given by the equation:

$$p(n.Ca) = \left(\frac{\frac{[Ca]}{K_1}}{1 + \frac{[Ca]}{K_1} + \frac{[Mn]}{K_2}}\right)^n \tag{2}$$

Because of the mean gradient of 3·73 (Table 2) obtained from log–log plots of m against [Ca], it will be assumed that n=4, and it will be shown that experimental results are consistent with this integral value of the exponent. The number of groups of four CaX's, which for convenience we will call 'quads' (quadruple calcium complexes), is equal to $p(4.Ca) \cdot X_T$ (where X_T is the total number of sites).

If the quantal content of e.p.ps is linearly related to the number of quads, then $m = Q \cdot p(4 \cdot Ca) \cdot X_T$ (where Q is the proportionality constant relating the number of quanta to the number of quads), i.e.

$$m = Q \cdot \left(\frac{\frac{[Ca]}{K_1}}{1 + \frac{[Ca]}{K_1} + \frac{[Mn]}{K_2}}\right)^4 \cdot X_T \tag{3}$$

Equation (3) can be written

$$m^{-\frac{1}{4}} = Q^{-\frac{1}{4}} \cdot \left(\frac{1}{[Ca]}\left(K_1 + \frac{K_1}{K_2} \cdot [Mn]\right) + 1\right) \cdot X_T^{-\frac{1}{4}} \tag{4}$$

It can be seen (after Lineweaver & Burk, 1934) that the intercept with the ordinate of a line relating $m^{-\frac{1}{4}}$ to $1/[Ca]$ (constant [Mn]) should be independent of [Mn] and that the slope should be a function of [Mn]. Results consistent with these predictions, based on the competition model, are shown in Figure 4. The reciprocal of the fourth root of quantal content was plotted against the reciprocal of calcium concentration at two values of [Mn]. The two straight lines meet at the ordinate and their slopes are related to [Mn]. In two of four similar experiments, the Mn lines met at the ordinate. In the other two, the lines met slightly to the right of the ordinate. These results confirm the hypothesis of competition between Ca and Mn in evoked transmitter release.

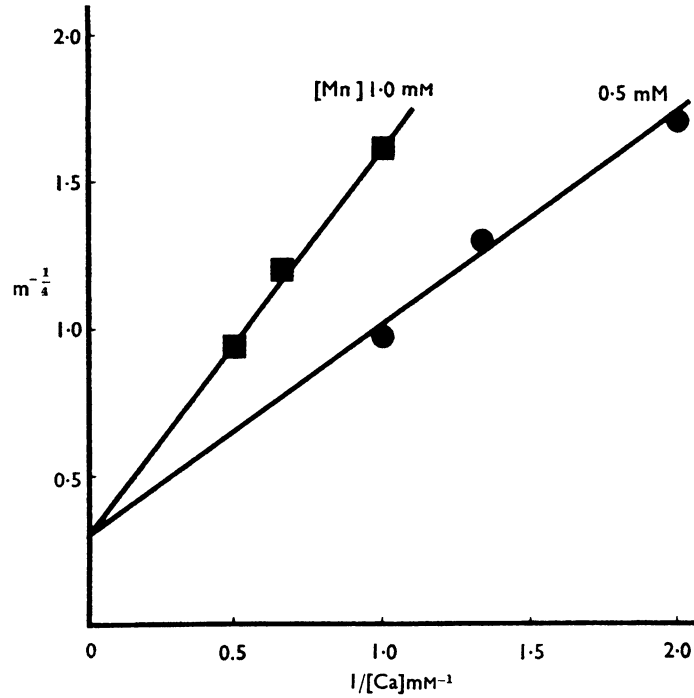

FIG. 4. Modified Lineweaver–Burk plot. The reciprocal of the fourth root of the average quantal content of endplate potentials ($m^{-\frac{1}{4}}$, ordinates) is plotted against the reciprocal of the calcium concentration (1/[Ca]mM^{-1}, abscissae) for solutions containing 1·0 mM Mn (squares), and 0·5 mM Mn (circles).

Estimations of dissociation constants for Ca and Mn, K_1 and K_2

If it is assumed that the proportionality constant, Q, of eqn. (3) is independent of Ca and Mn concentrations, a series of values for quantal content, [Ca] and [Mn] can be substituted in the equation, and the simultaneous equations then solved for K_1 and K_2. This was done, but it was clear that very small variations in m gave large variations in the calculated values of K_1 and K_2, partly because of the fourth power relationship. However, it was still thought worthwhile to obtain values for the dissociation constants while remaining aware of possible errors. Dissociation constants were calculated for ten preparations in which quantal contents had been measured from large numbers of e.p.ps. The value for K_1 varied from 0·3 to 3·3 mM with a mean value of 1·34 (S.E.M.=0·33). The value of K_2 varied from 0·02 to 0·26 mM with a mean value of 0·145 mM (S.E.M.=0·026). In spite of the wide ranges, it was apparent that the affinity for Mn was significantly higher than for Ca.

Returning to the relationship between m and [Mn], it is possible to use the values for K_1 and K_2 to test a fourth power relationship for the denominator of equation (3), with variations of [Mn] and a constant [Ca]. The mean values of K_1 and K_2 were used (1·3 and 0·15 mM) to plot log m against log $(1+[Ca]/K_1+[Mn]/K_2)$. The gradients obtained from the results of ten experiments ranged from −3·3 to −4·8 mM (Table 3) with a mean value of −3·94 mM (S.E.M.=0·15) which is close to the expected value of 4.

TABLE 3. *Gradients of lines relating log quantal content to log* $(1+[Ca]/K_1+[Mn]/K_2)$

Cell	Ca (mM)	Mn range (mM)	Gradient
1	1·80	1·40–2·20	−3·6
2	1·80	0·80–1·40	−4·8
3	1·80	1·20–1·80	−3·9
4	1·80	1·20–1·60	−4·3
5	0·75	0·60–1·13	−4·5
6	0·75	1·00–1·25	−4·0
7	0·75	0·50–0·90	−3·3
8	0·50	0·25–0·63	−4·0
9	0·50	0·25–0·75	−3·5
10	0·50	0·25–0·63	−3·5
			mean=3·94
			S.E.M.=0·15

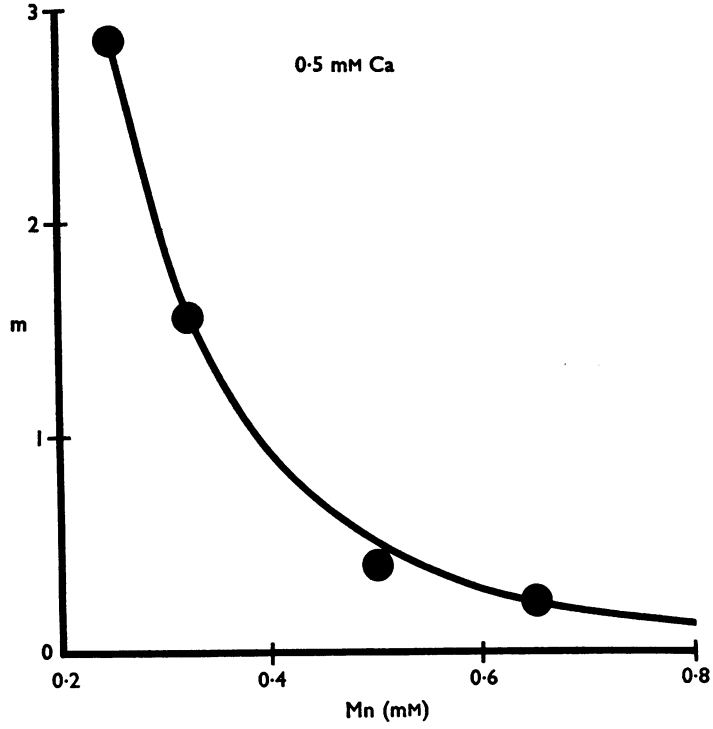

FIG. 5. Average quantal content (m) plotted against manganese concentration ([Mn]). Solutions contained 0·5 mM Ca. The line was calculated from eqn. (3) with K_1=1·3 mM and K_2=0·15 mM, and a proportionality constant chosen to superimpose observed and calculated quantal contents (m, ordinates) at [Mn]=0·25 mM.

The mean values of K_1 and K_2 gave reasonably good fits to experimentally obtained data (filled circles, Fig. 5) recorded in experiments in which [Mn] was varied. The curved line in Fig. 5 was obtained by calculating values for m from equation (3), with K_1 and K_2 set at 1·3 and 0·15 mM respectively. A proportionality constant, obtained from the ratio of the recorded m to the calculated m at [Mn]= 0·25 mM, was used to superimpose the calculated curve on the experimental points. The line in Fig. 2 was obtained in the same way and in both Fig. 2 and Fig. 5, the experimental points fit the lines obtained from equation (3) rather well.

Effect of Mn on spontaneous release of transmitter

In contrast to its effect on evoked release, Mn caused a delayed increase in m.e.p.p. frequency. The increase in frequency depended on the concentrations of Mn and Ca, and the duration of exposure to the solution. In Figure 3B, results obtained in a preparation exposed to a solution containing 2 mM Mn and 1·8 mM Ca are shown. The bars show ± 1 S.E. of the mean frequency of m.e.p.ps. The increase in frequency was not immediate but began after about 30 minutes. The change in frequency was progressive and varied widely from fibre to fibre. The influence of the concentration of calcium ions on the increase in m.e.p.p. frequency caused by Mn can best be illustrated by an example. The increase in frequency of m.e.p.p.s in a fibre exposed to a solution containing 1·8 mM Mn and 1·8 mM Ca was 400% after two hours. In the same fibre a solution containing 1·8 mM Mn but 1 mM Ca caused only a 20% increase in m.e.p.p. frequency after the same time. In general, the increase in frequency caused by Mn was reduced by lowering the Ca concentration.

When the release of quanta of transmitter was stimulated by raising the potassium concentration, manganese ions depressed m.e.p.p. frequency (cf. Kajimoto & Kirpekar, 1972). For example, m.e.p.p. frequency increased from 0·23 to 2·82 per second in a solution containing 15 mM K^+, then fell to 0·44 per second in a solution containing 15 mM K^+ and 2 mM Mn^{++}.

Discussion

Manganese has a powerful depressant effect on evoked transmitter release. The results obtained are consistent with the hypothesis that there is competition between calcium and manganese in excitation–secretion coupling, and that the site at which they compete has a higher affinity for manganese than for calcium. The results are similar to those obtained for magnesium which also competes with calcium in excitation–secretion coupling (Jenkinson, 1957; Dodge & Rahamimoff, 1967; Hubbard *et al.*, 1968b) but are different in that manganese is a much more effective competitor than magnesium. Thus, evoked transmitter release can be reduced to low levels with very little manganese (0·5–2 mM) and, hence, with little disturbance of osmotic pressure or ionic strength in solutions used in experiments.

The results are in good agreement with the model (eqn. (3)) but give no evidence as to the site where calcium and manganese act. A quad may represent a critical area of membrane which must be activated before a quantum of transmitter can be released; or it may represent a depolarization-activated 'channel' which allows calcium ions to enter the nerve terminal only when four calcium ions are 'lined up' in the channel. What is fairly certain is that evoked transmitter release is related to the probability of an event which involves more than one, and probably four, calcium ions.

It has been suggested that in some preparations, the cooperative action of three calcium ions (Hubbard *et al.*, 1968b; Balnave & Gage, 1971) rather than four (Dodge & Rahamimoff, 1967) underlies transmitter release. These conclusions have been based on log–log plots of quantal content (or postsynaptic potential amplitude) against [Ca] or [Mn], in which slopes as low as 2·6 can be obtained (Hubbard *et al.*, 1968; Katz & Miledi, 1970). However, these gradients are predicted by a fourth power relationship between transmitter release and calcium concentration. This is

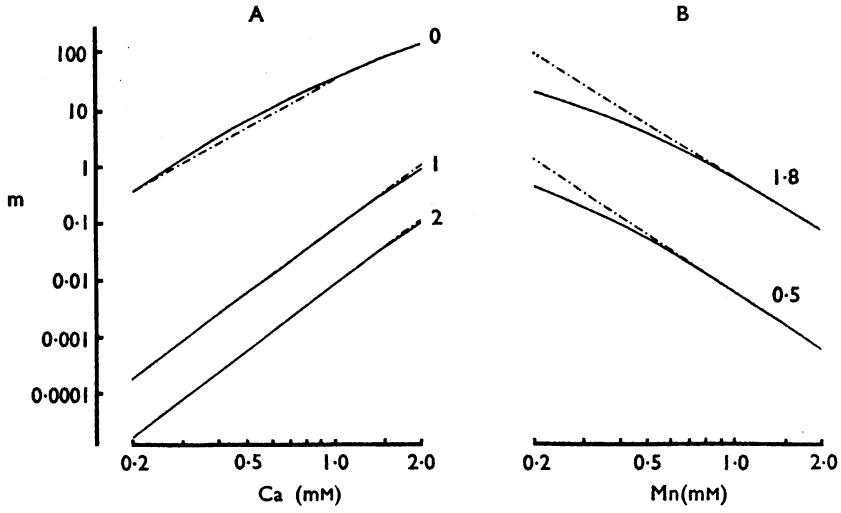

FIG. 6. Curves (continuous lines) calculated from equation (3) relating quantal content (m) to calcium (A) or manganese (B) concentrations. The broken lines are fitted to parts of the curves (see text) to obtain gradients. The numbers next to the curves denote the background manganese (A ; 0, 1 and 2) and calcium (B ; 1·8 and 0·5) concentrations in mmol/litre. Note the logarithmic axes. Ordinates: quantal content, m, obtained by using an arbitrary but reasonable value for the proportionality constant, Q, in equation (3).

illustrated in Figure 6. Using eqn. (3) with the exponent of 4 (with K_1=1·3 mM, K_2=0·15 mM, and with an arbitrary value for Q to give values of m consistent with experimental results), curves relating m to [Ca] were calculated (Fig. 6A). The three curves from above down, show the calculated relationship (note the logarithmic axes) in the presence of 0, 1 and 2 mM manganese respectively. In the absence of manganese, the curve is clearly not linear. However, a straight line drawn through the curve at Ca=0·2 mM and Ca=1 mM has a gradient of 2·4 (cf. Fig. 2, Hubbard *et al.*, 1968b). The curves obtained with Mn concentrations of 1 and 2 mM are reasonably linear, at least for [Ca] $\leqslant$1·5 mM. The broken lines fitted to the left parts of the curves have gradients of 3·7 (middle curve) and 3·85 (lowest curve) which are close to the mean gradient of 3·73 obtained here.

Balnave & Gage (1971) suggested a third power relationship between transmitter release and manganese concentration because of a mean gradient of about 3·3 obtained from log–log plots of m against [Mn]. Again, such a gradient is predicted by a fourth power relationship. This is shown in Fig. 6B, in which curves of the relationship between m and [Mn] have been calculated from eqn. (3) (with its exponent of four) using dissociation constants and proportionality constant, Q, as before. The two curves, corresponding to calcium concentrations of 1·8 mM (above) and 0·5 mM (below) become more markedly curved at lower Mn concentrations. Straight lines are drawn through the curves in the region of 1 to 2 mM, as was done with experimental results on similar log–log plots. The gradients of the two lines are −3·2 above, and −3·4 below, which are very close to the mean slope of −3·3 obtained from experimental results. Hence the gradients of log–log plots of quantal content against [Ca], and against [Mn], are more consistent with a fourth than with a third power.

Because of the fourth power of the relationship and unavoidable errors in the estimation of m, it is difficult to calculate K_1 and K_2 with a great degree of confidence.

either by using simultaneous equations or by equating equal quantal contents. However, the mean values obtained here for K_1 and K_2 do not appear unreasonable. Dodge & Rahamimoff (1967) obtained a mean value of 1·1 mM for K_1, which is reasonably close to the value of 1·3 mM calculated above. A dissociation constant of 0·15 mM for Mn is also consistent with the relative effects of Mn and Mg on transmitter release. The inhibitory effects of 20 mM Mg and 1–2 mM Mn are roughly equivalent (Balnave & Gage, unpublished observations). It would be expected, therefore, that the dissociation constant of Mn would be about one-tenth to one-twentieth of the dissociation constant for Mg which has been reported to be approximately 3 mM (Dodge & Rahamimoff, 1967). The best evidence that the values for the dissociation constants are not unreal are the fits to observed e.p.ps shown in Figs. 2 and 5. However, it should be mentioned that the values of K_1 and K_2 were derived by substitution of m, [Ca] and [Mn] in equation (3). If a better model is found, K_1 and K_2 may have to be recalculated. For example, for the sake of simplicity, a model in which four (or more) calcium ions bind to X has not been used because it would be necessary to use four dissociation constants rather than one, and the power relationship could not be determined simply from log–log plots. As it is, there seems no reason to reject the model adopted here (cf. Dodge & Rahamimoff, 1967) while it is so consistent with the experimental results.

It is possible that the total number of 'receptor sites', X_T, may impose a real restriction on the amount of transmitter which can be released by an action potential. If one quad causes the release of only one quantum of transmitter ($Q=1$, eqn. (3)), the maximum number of 'receptor sites' per nerve terminal must be of the order of 10^3 to 10^4. Furthermore, if a quad causes the release of more than one quantum of transmitter (i.e. $Q>1$), the maximum number of sites would be even less. If a quad becomes inactive for some time after being active (e.g. by a process of 'calcium inactivation', similar to the inactivation of sodium conductance which occurs during an action potential), X_T could conceivably be appreciably depleted during repetitive activity, and this would lead to depression of transmitter release. Nerve terminals in the sartorius muscle of the toad vary from 100 μm to 500 μm (or more) in length and are approximately cylindrical, with a diameter of about 1 μm (Gage, unpublished observations). If calcium receptor sites are dispersed evenly over the surface of a terminal there must be fewer than 30 such sites per square micron of membrane.

In contrast to its effect on the release of transmitter by an action potential, manganese stimulates spontaneous release. Lanthanum has similar paradoxical actions (Blioch, Glagoleva, Liberman & Nenashev, 1968; Heuser & Miledi, 1971; De Bassio, Schnitzler & Parsons, 1971; Kajimoto & Kirpekar, 1972) though its effects on m.e.p.p. frequency are much more dramatic. Kajimoto & Kirpekar did not observe an increase in m.e.p.p. frequency with Mn, presumably because the effect is delayed and their duration of observation was only 20 minutes. The relationship between m.e.p.p. frequency and [Ca] does not conform to a fourth power relationship (Del Castillo & Katz, 1954a; Hubbard, 1961; Gage & Quastel, 1966; Hubbard, Jones & Landau, 1968a), so quads are probably not required for the spontaneous release of transmitter. It seems likely, therefore, that quads are only associated with the influx of calcium into a nerve terminal through depolarization-activated calcium 'channels'. Manganese (and other competitive inhibitors of release) probably reduce this depolarization-activated calcium influx by reducing

the probability of formation of quads. Therefore, they might not be expected to reduce m.e.p.p. frequency which does not seem to depend on quads. On the other hand, manganese may enter a nerve terminal and displace calcium from an internal store (e.g. by competing with calcium for a chelating ' buffer ') and hence, by raising the intracellular concentration of ionized calcium, increase m.e.p.p. frequency.

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