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CALCIUM DEPENDENCE OF EVOKED TRANSMITTER RELEASE AT VERY LOW QUANTAL CONTENTS AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. The relationship between the rate of evoked transmitter release and the extracellular concentration of Ca ions, $[Ca^{2+}]_o$, was studied at surface neuromuscular junctions of the frog cutaneous pectoris muscle. The average quantal content of the end-plate potential was reduced to low levels by reducing $[Ca^{2+}]_o$ and adding 2 mM-Mn^{2+}, 4 mM-Co^{2+} or 10 mM-Mg^{2+}.

2. When the motor nerve was stimulated at a low frequency (0·5–2 Hz) in 2 mM-Mn^{2+} or 4 mM-Co^{2+}, the average quantal content of evoked release was proportional to the fourth power of $[Ca^{2+}]_o$ down to the lowest measurable quantal contents, around 2–4 quanta per 1000 stimuli. Combined with previous studies, this result indicates that evoked transmitter release has a steep, nonlinear dependence on $[Ca^{2+}]_o$ over four orders of magnitude of evoked release.

3. Calculations predict that if evoked and spontaneous release have the same fourth power dependence on intracellular $[Ca^{2+}]$, then the curve relating evoked release and $[Ca^{2+}]_o$ should become much less steep as the evoked release rate approaches the spontaneous release rate. Our observation that the relationship between evoked release and $[Ca^{2+}]_o$ remains fourth power down to very low release rates suggests that most spontaneous quantal release does not have the same dependence on intracellular $[Ca^{2+}]$, or does not use the same intracellular Ca^{2+} pool, as evoked release.

4. In 2–10 mM-Mg^{2+}, the lowest average quantal contents were markedly higher than the fourth power prediction. This discrepancy may occur either because Mg^{2+} somehow elevates intracellular $[Ca^{2+}]$, or because Mg^{2+} is itself a weak activator of transmitter release.

5. Even at very low rates of evoked release, increasing the stimulus frequency to 5–50 Hz caused a progressive increase in both evoked release and the rate of 'background' quantal release during the interstimulus interval. The frequency-dependent enhancement of both evoked and background release was more pronounced in solutions containing 10 mM-Mg^{2+} than in solutions containing 2 mM-Mn^{2+}.

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INTRODUCTION

At the neuromuscular junction (and other chemically mediated synapses) an influx of Ca ions is essential for the quantal transmitter release evoked by nerve stimulation. In an attempt to learn more about the stoichiometry of the relationship between calcium and release, numerous studies have examined how evoked release varies with the extracellular Ca concentration, $[Ca^{2+}]_o$. At the frog neuromuscular junction the relationship between release (measured as the amplitude or quantal content of the end-plate potential) and $[Ca^{2+}]_o$ is highly nonlinear (Jenkinson, 1957; Dodge & Rahamimoff, 1967). The steepest part of this relationship occurs at low release rates (average quantal content 0·5–15), where Dodge & Rahamimoff (1967) found that double logarithmic plots of evoked release *vs.* $[Ca^{2+}]_o$ have an average slope of 3·78. This result suggested to them that four Ca ions may be required to activate a release site. Crawford (1974) examined the relationship between evoked release and $[Ca^{2+}]_o$ at even lower quantal contents, and found a rather sudden transition from a fourth power to an approximately linear relationship at quantal contents around 0·015–0·65. This finding suggested that under some conditions a single Ca ion may suffice to activate a release site.

We re-examined the relationship between release and $[Ca^{2+}]_o$ in the range of low quantal contents, and found that this relationship remains fourth power down to the smallest evoked release rates we could measure (2–4 quanta per 1000 stimuli), provided that the motor nerve is stimulated at a constant slow rate (0·5–2 Hz) and that Mn^{2+} or Co^{2+} is used as the Ca^{2+} antagonist. We found that at these low release rates both Mg^{2+} (2–10 mM) and modest elevations of stimulus frequency (up to 5–20 Hz) increase the quantal content of the end-plate potential, findings that help explain the deviation from fourth power reported by Crawford (1974).

Some of these results were published in abstract form (Andreu & Barrett, 1979).

METHODS

Preparation and solutions. Experiments used the cutaneous pectoris nerve-muscle preparation from small *Rana pipiens* (northern variety). Much of the connective tissue covering the muscle's dorsal surface was carefully removed. Surface end-plates were located using a compound microscope (sometimes using polarized light or Hoffman modulation-contrast optics to increase contrast), and standard 10–20 MΩ micropipettes filled with 0·25 M-K citrate were inserted into the underlying muscle fibre about 20 μm from the end-plate. The motor nerve was stimulated with brief suprathreshold pulses via a suction electrode.

The most straightforward way to achieve the desired reduction in the quantal content of the end-plate potential (e.p.p.) is simply to reduce $[Ca^{2+}]_o$ to very low levels using suitable Ca buffers (citrate, EGTA). This simple technique was unacceptable for our experiments because in $[Ca^{2+}]_o$ below about 0·5 mM the muscle membrane tended to seal poorly around the electrode, and miniature end-plate potential (m.e.p.p.) and e.p.p. amplitudes would have declined substantially during the prolonged (> 1 hr) recording periods required for reliable measurement of low quantal contents during low frequency nerve stimulation. Instead we brought quantal contents into the desired low range by adding to the bath 2–4 mM-Mn^{2+}, 2–4 mM-Co^{2+} or 2–10 mM-Mg^{2+}. Lower concentrations of Mn^{2+} and Co^{2+} were used because these ions block Ca^{2+}-dependent release more efficiently than Mg^{2+} (Meiri & Rahamimoff, 1972; Balnave & Gage, 1973; Weakly, 1973; Crawford, 1974), and because higher concentrations of Mn^{2+} and Co^{2+} tend to block nerve conduction (our observation; see also Baker, Meves & Ridgway, 1973). Use of these divalent cations allowed us to achieve the desired low quantal contents while still keeping $[Ca^{2+}]_o$ above

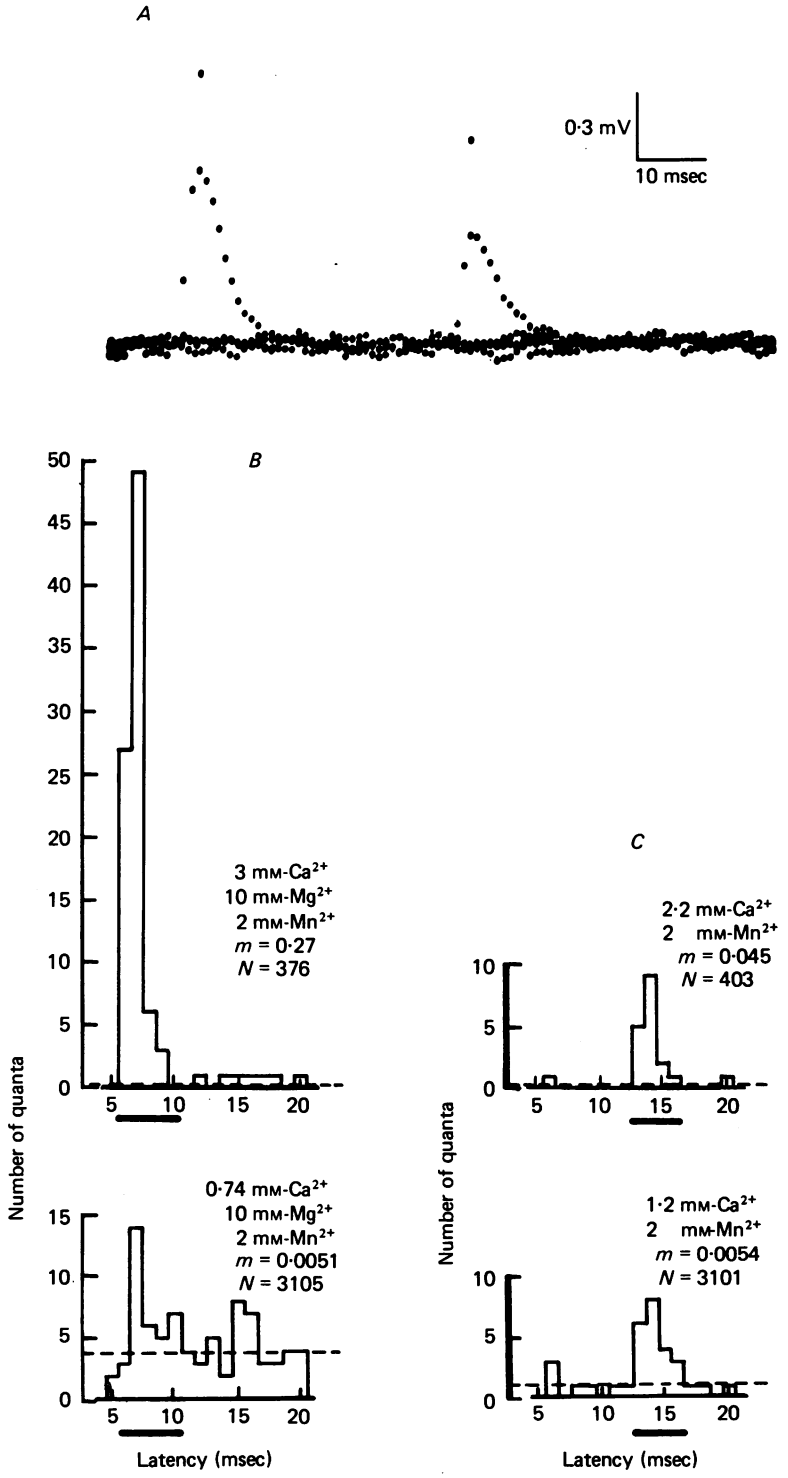
0·5 mM. Solutions containing Mn^{2+} were especially satisfactory, yielding large, stable resting potentials and low, stable resting m.e.p.p. frequencies, allowing recording for up to 7 hr. We added 0·05 mM-ascorbic acid (a reducing agent) to solutions containing Mn^{2+} to retard the oxidation of Mn^{2+} to Mn^{3+} and the resultant precipitation of $Mn(OH)_3$. Mg ions did not stabilize the preparation as well as Mn^{2+} or Co^{2+}. In our hands resting potentials and m.e.p.p. amplitudes often diminished rapidly in Ringer solutions containing only low $[Ca^{2+}]$ and high $[Mg^{2+}]$. (The loss of resting potential may have been due to poor sealing of the muscle fibre membrane around the electrode tip.) Thus the effects of Mg^{2+} were usually studied by adding Mg^{2+} to Ringer solutions containing 2 mM-Mn^{2+}.

Solutions were prepared during the course of each experiment from stock solutions containing either no divalent cations, 10 mM-$MnCl_2$, 10 mM-$CoCl_2$ or 20 mM-$MgSO_4$. All stock solutions contained 112 mM-NaCl, 2 mM-KCl, 3 mM-glucose, 2 mM of the H^+ buffer PIPES (piperazine - N, N′-bis (2-ethanesulphonic acid)) and 0·01 mM-phenol red. The pH of all solutions was adjusted to 7·3 by titration against a phenol red colorimetric standard. The osmolarity of the stock solutions, tested with a Fiske osmometer, was generally within 5 % of the mean for frog plasma, 243 m-osmole (Fenn, 1936). The preparation was continuously perfused at 0·3–2 ml./min, and maintained at 20 ± 0·5 °C with a Peltier-effect thermoelectric module. Following solution changes, 15–30 bath volumes were passed through the experimental chamber before quantal contents were measured.

Experimental protocol. The most rigorous way to test the relationship between e.p.p. quantal content (m) and $[Ca^{2+}]_o$ is to measure quantal contents at a variety of $[Ca^{2+}]_o$ in a single preparation. This technique is feasible for quantal contents exceeding 0·1, but is difficult at lower quantal contents, where thousands of stimuli must be given to obtain a reliable estimate of m. Crawford (1974) met this problem by stimulating faster (up to 10 Hz) at lower quantal contents, but in our hands these higher frequencies progressively increased m (e.g. Fig. 6), so we felt constrained to keep the same low stimulus frequency (0·5–2 Hz, or 1800–7200 stimuli/hr) throughout the range of quantal contents in a given preparation. At these low stimulus frequencies a single determination of a very low m value (e.g. $2–4 \times 10^{-3}$) took 1–2 hr, so we could measure at most two low m values per preparation. Because of this constraint, most experiments used the following basic strategy (see e.g. Fig. 2): Ca^{2+} and Mn^{2+} (or Co^{2+} or Mg^{2+}) concentrations were adjusted to give an average quantal content in the range 0·1–1. This m was measured by administering several hundred stimuli at 0·5–2 Hz, and entered as one point on a double logarithmic plot of m *vs.* $[Ca^{2+}]_o$. A line with a slope of 4 was drawn through this point and used to choose a $[Ca^{2+}]_o$ expected to give an m of $2–6 \times 10^{-3}$ if the fourth-power hypothesis were true (m would exceed this value if release becomes a linear function of $[Ca^{2+}]_o$ at low m). We aimed for these very low m values to maximize the difference between the m values predicted by the fourth-power model of Dodge & Rahamimoff (1967) and the linear model of Crawford (1974). After equilibrating the preparation for at least 0·5 hr at this new low $[Ca^{2+}]_o$, we administered several thousand stimuli at 0·5–2 Hz to determine m. In most experiments we then returned to the original $[Ca^{2+}]_o$ and redetermined m to check for drift. If the preparation remained stable, we either measured m at higher $[Ca^{2+}]_o$, or returned to the low $[Ca^{2+}]$ solution to determine m at higher frequencies (5–20 Hz).

Detection of quantal potentials. During the course of the experiment all quantal potentials, both evoked and spontaneous, were detected by a PDP 11-10 digital computer, which also controlled the stimulation pattern. The detection programme, written by K. Magleby and F. Morris, used adjustable amplitude, rate of rise and rate of decay criteria to detect e.p.p.s. and m.e.p.p.s (Fig. 1*A*). The accuracy of detection was continuously monitored by watching a computer-controlled oscilloscope display of both the voltage record and dots indicating quantal detection. Detection parameters were tailored to each neuromuscular junction so that the rate of detection failures (false negatives) or erroneous detections (false positives) was well under 2 %. The peak amplitude and latency of each detected e.p.p. and m.e.p.p. were stored for subsequent analysis.

Measurement of e.p.p. quantal content. Histograms of post-stimulus quantal latencies (e.g. Fig. 1*B*, *C*) were constructed, and used to define the evoked release interval, the 3–5 msec post-stimulus interval bracketing the peak quantal release rates. Data of Katz & Miledi (1965) and Barrett & Stevens (1972*b*) indicate that at 20 °C an interval of this duration should encompass most phasic (but not delayed) evoked release. At a given junction the duration of the evoked release interval was kept constant for all $[Ca^{2+}]_o$, because histograms like those of Fig. 1*B*, *C*


A
0·3 mV
10 msec
B
3 mM-Ca2+
10 mM-Mg2+
2 mM-Mn2+
m = 0·27
N = 376
0·74 mM-Ca2+
10 mM-Mg2+
2 mM-Mn2+
m = 0·0051
N = 3105
Number of quanta
Latency (msec)
C
2·2 mM-Ca2+
2 mM-Mn2+
m = 0·045
N = 403
1·2 mM-Ca2+
2 mM-Mn2+
m = 0·0054
N = 3101
Number of quanta
Latency (msec)

indicate that changes in $[Ca^{2+}]_o$ and quantal content over the ranges studied here have relatively little effect on the (amplitude-normalized) time course of phasic evoked release. At some junctions the peak of the post-stimulus latency histogram shifted 1–2 msec later over the course of a long experiment, necessitating a corresponding shift in the position of the evoked release interval. These shifts were not necessarily due to changes in $[Ca^{2+}]_o$ or quantal content, since similar shifts in e.p.p. latency also occur over time in preparations bathed in a constant $[Ca^{2+}]_o$ (Barrett & Stevens, 1972*a*).

All quantal contents were corrected for the rate of 'background' or spontaneous release. This correction was especially important when the evoked release rate was very low. The background release rate was measured from the number of quanta released during a series of late post-stimulus intervals, e.g. between 500 and 997 msec following each impulse during stimulation at 1 Hz, or between 50 and 97 msec during 10 Hz stimulation. At low stimulus frequencies this background release rate was usually stable for a given $[Ca^{2+}]_o$, but it often increased progressively during higher frequency stimulation, especially in solutions containing Mg^{2+} or Co^{2+} (e.g. Fig. 6). Delayed release, a slowly decaying elevation of m.e.p.p. frequency following the e.p.p. (Rahamimoff & Yaari, 1973), was present at some junctions, but had usually dropped to low levels during the interval in which background release rates were determined.

The number of quanta released during the evoked release interval was corrected for background release using this equation:

$$x_E = x_T - x_B, \tag{1}$$

where x_E is the (corrected) number of evoked releases, x_T is the total number of releases observed in the evoked release interval, and x_B is the product of the background release rate times the evoked release interval. Assuming that evoked and background quantal releases are random, noninteracting Poisson processes, the standard error of x_E is given by (see Baird, 1962):

$$\text{S.E. } x_E = \sqrt{x_T + x_B} \tag{2}$$

For example, if in 1600 trials a total of 25 quantal releases occurred during the 10 msec evoked release interval, and 450 releases occurred during the 500 msec background release interval, then an average of 9 background releases would be expected to have occurred during the evoked release interval, leaving an estimate of 16 evoked releases. The standard error of this estimate, S.E. x_E, is equal to $\sqrt{34}$, or about 5·8. Compared to the large relative error in measuring evoked release at low quantal contents, the relative error involved in measuring the average background release rate was usually small, because in the prolonged trains used to determine low quantal contents, the total number of background releases counted usually exceeded 250.

The average quantal content (m) of evoked release was calculated in three standard ways (Martin, 1966), all corrected for background release rates. These three methods usually gave similar values for m. The simplest method, accurate when evoked release rates are so low that it is very unlikely that more than 1 quantum will be released during the evoked release interval, is simply to divide x_E (eqn. 1) by the number of stimuli, N. The standard error of this estimate of

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Fig. 1. *A*, superimposed voltage traces showing detection of spontaneous m.e.p.p.s by the quantal detection computer program. Voltage is sampled at 1 msec intervals, and the program searches for m.e.p.p.s and e.p.p.s using criteria for amplitude, rate of rise and rate of decay. The program indicates detection by placing a dot over the peak of the potential, and stores the latency and peak amplitude of that potential for future analysis. *B*, paired post-stimulus latency histograms measured at one junction in two different $[Ca^{2+}]_o$ in 2 mM-Mn^{2+}, 10 mM-Mg^{2+} during 1 Hz stimulation. Latency is the interval between nerve stimulation and quantal detection. Bars indicate the evoked release interval, 6–10 msec. At this junction the period of maximal release did not appear to vary with $[Ca^{2+}]_o$ or quantal content. Horizontal dashed lines indicate quanta expected due to background release. There are more background quanta at low $[Ca^{2+}]_o$ because more total trials (N) were sampled to determine quantal content at low $[Ca^{2+}]_o$. Evoked release (m) was measured by subtracting this background release from the total release occurring during the evoked release interval. *C*, as in *B*, except solutions contained no Mg^{2+}, and the evoked release interval was 13–16 msec. The evoked release interval begins later than in *B* because the time required for nerve conduction was longer.

quantal content is simply S.E. x_E/N. In the numerical example given above, m is 16/1600, or 0·01, with a standard error of $\sqrt{(34)}/1600$, or 0·0036. (Standard errors were a smaller percentage of the mean at higher quantal contents.) Another method, applicable to quantal contents below about 2·5, uses Poisson's theorem to calculate m as the natural logarithm of the ratio of the total number of stimuli divided by the number of evoked release intervals during which no quantal potentials occurred ('failures'). The third method, applicable at virtually all m, determines m from the ratio of the average e.p.p. amplitude divided by the average amplitude of a spontaneous quantal potential. Where necessary, e.p.p. amplitudes were corrected for nonlinear summation of quantal potentials by averaging Martin's (1955) and Stevens' (1976) corrections. During high frequency (5–50 Hz) stimulation m tended to increase with time, especially in solutions containing Co^{2+} or Mg^{2+} (e.g. Fig. 6). Values of m plotted in the Figures were averaged over the entire period of stimulation.

RESULTS

Experiments in Mn^{2+}

Fig. 2 is a double logarithmic plot of the relationship between evoked release and $[Ca^{2+}]_o$ obtained in 2 mM-Mn^{2+} at one neuromuscular junction. It illustrates our typical finding that the average quantal content is proportional to the fourth power of $[Ca^{2+}]_o$ down to very low release rates when all quantal contents are measured at the same low rate of stimulation, but that the relationship deviates from fourth power with higher stimulation frequencies. In this experiment the initial solution contained 2 mM-Mn^{2+} and 2·2 mM-Ca^{2+}. In 404 trials at a stimulation rate of 2 Hz there were seventy-eight successes during the 3 msec evoked release interval, only 1·6 of which were attributable to the background release rate of 1·3/sec. The average quantal content, estimated from both failures and the e.p.p./m.e.p.p. amplitude ratio, was 0·19 (point 1). Bath $[Ca^{2+}]$ was then lowered to 0·8 mM, and 2722 stimuli were delivered at 2 Hz. A total of 19 quanta were released during the 3 msec evoked release interval, 9·3 of which were attributable to the background release rate of 1·14/sec. An average quantal content of $0·0036 \pm 0·0020$ (point 2) was calculated from the remaining 9·7 evoked quanta, an m value very close to the fourth power prediction (line) extrapolated from point 1. The preparation was then returned to the initial 2·2 mM-Ca^{2+} solution, where a redetermination of m (0·21, point 3) showed little drift. Bath $[Ca^{2+}]$ was once again lowered to 0·8 mM and the preparation was stimulated 2023 times at a higher frequency, 10 Hz. The elevated stimulus frequency increased the mean background release rate to 3·16/sec, and increased m (corrected for background release) to $0·0125 \pm 0·0039$ (point 4), a value more than three times higher than the fourth power prediction for this $[Ca^{2+}]$. Subsequent determinations of m at 2 Hz in the original 2·2 mM-Ca^{2+} (0·164, point 5) and in 3 mM-Ca^{2+} (0·76, point 6) were again compatible with the fourth power hypothesis. Thus in this preparation evoked release was proportional to the fourth power of $[Ca^{2+}]_o$ over more than two orders of magnitude of release rate, down to an average quantal content of only 0·0036, provided that the same low stimulus frequency (2 Hz here) was used throughout. A moderate increase in stimulus frequency (to 10 Hz) increased the low $[Ca^{2+}]$ value of m to well above its predicted fourth power value.

Fig. 3 summarizes the low m results of nine experiments similar to that of Fig. 2 performed in 2 mM-Mn^{2+}. Part *A* shows m values determined at low stimulus frequencies (0·5–2 Hz), part *B* at higher frequencies (5–50 Hz). At low stimulus fre-

quencies (part *A*) most of the data points fall fairly close to the fourth power prediction (continuous line). The standard error associated with each of these very low *m* values is relatively large, 31–119 % of the mean for the *m* values in Fig. 3*A*. Many of the quanta released during the 3–5 msec evoked release interval following stimulation were attributable to background release, and *m* values were calculated from

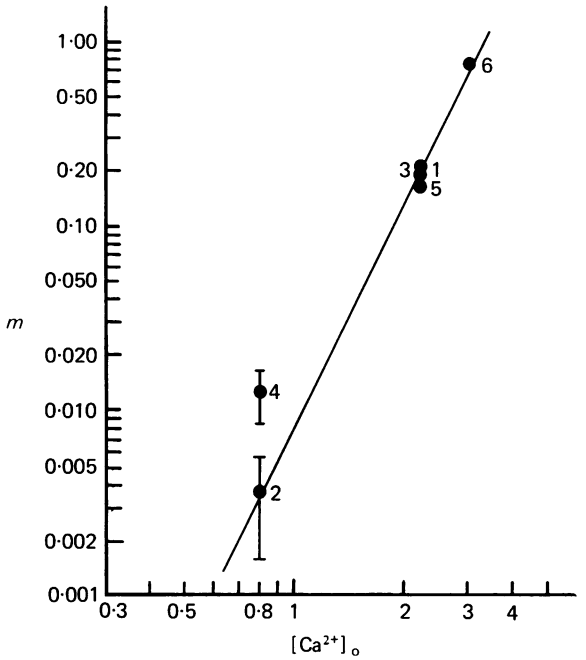

Fig. 2. Double logarithmic plot of average quantal content (*m*) as a function of bath $[Ca^{2+}]$ for a neuromuscular junction bathed in Ringer solutions containing 2 mM-Mn^{2+}. Numbers near the data points indicate the order in which $[Ca^{2+}]_o$ was changed. Point 4 was measured during stimulation at 10 Hz. The other points, measured during 2 Hz stimulation, fall near the fourth power prediction (line) drawn through point 1. Quantal contents, measured during a 3 msec evoked release interval, were all corrected for background release at the appropriate $[Ca^{2+}]_o$ and stimulation frequency (see text and Methods). The bars bracketing points 2 and 4 indicate the standard error of the average quantal content, calculated using eqn. (2). Standard errors for points 1, 3 and 5 were about 0·02; that for point 6 was 0·04.

the remaining evoked quanta (5·5–22·4 in 1850–3100 trials for the *m* values in Fig. 3*A*). However, although each individual low *m* value is subject to considerable error, the results of all the low frequency experiments in Mn^{2+} taken together do support the fourth power hypothesis. The data are certainly fitted better by the fourth-power relationship (continuous line) than by linear relationships (dashed lines) drawn through the average or even the lowest 'transition' quantal content reported by Crawford (1974). Thus it appears that the fourth power relationship between $[Ca^{2+}]_o$ and evoked release persists down to average quantal contents as low as 0·002 in 2 mM-Mn^{2+}. The low frequency, low *m* value that deviates most markedly

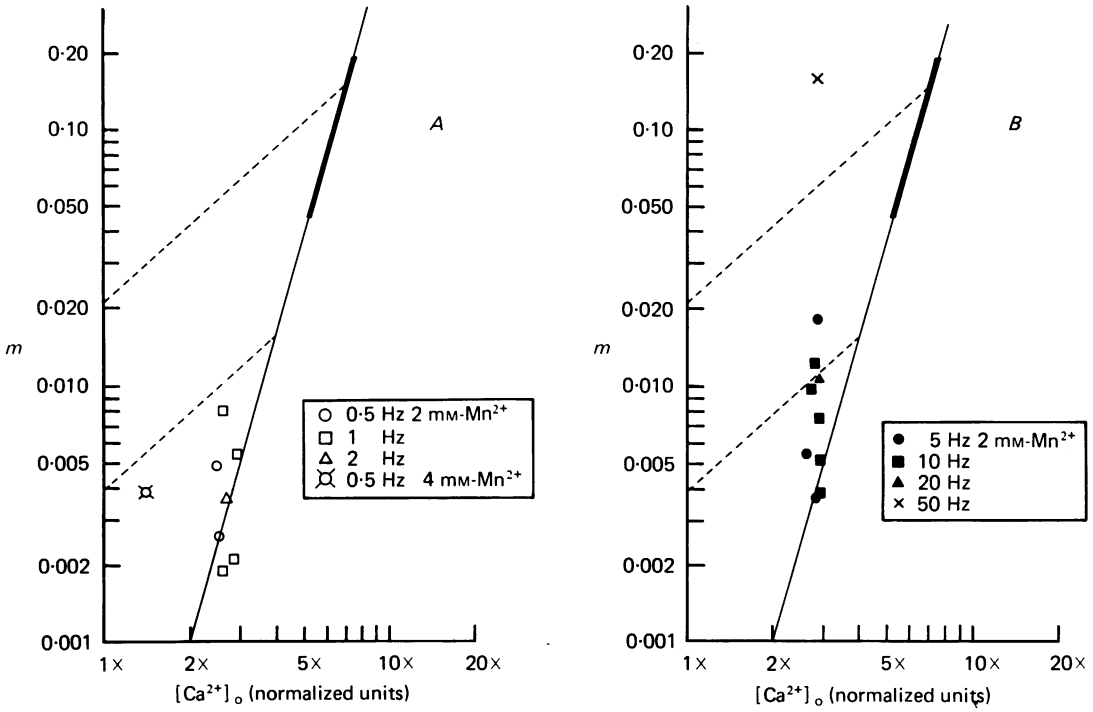

Fig. 3. Double logarithmic plot of average quantal content (m) *vs.* $[Ca^{2+}]_o$ (normalized units), summarizing results obtained in 2 mM-Mn^{2+} (4 mM in one case ⌑) and $[Ca^{2+}]_o$ ranging from 0·5 to 1·23 mM. Part *A* shows m values measured at low frequencies (0·5–2 Hz); part *B* at higher frequencies (5–50 Hz). To allow comparison of results from different junctions, all data points are plotted relative to the same fourth-power prediction (continuous line). This normalization was achieved by displacing all m values from a given junction laterally along the $[Ca^{2+}]_o$ axis until the initial m value (analogous to point 1 in Fig. 2) lay on a single reference line with a slope of 4. The thickened portion of this fourth power line indicates the range of initial m values in 2 mM-Mn^{2+} (0·045–0·19 in $[Ca^{2+}]_o$ between 1·6–2·2 mM). All m values were corrected for the background release rate at that stimulus frequency. Note that higher stimulation frequencies (part *B*) produce higher m values. The dashed lines represent a linear relationship between $[Ca^{2+}]_o$ and release, drawn through the lowest (m = 0·015) and average (m = 0·15) 'transition' quantal contents reported by Crawford (1974). To avoid confusion, standard error bars are not included; they were of the same order of magnitude as those in Fig. 2.

from the fourth power prediction was determined in 4 mM-Mn^{2+} instead of the usual 2 mM.

Fig. 3*B* shows that during higher frequency stimulation (5–50 Hz) most quantal contents exceed the fourth power prediction. These summed results, plus paired results from junctions where two stimulation frequencies were tested in low $[Ca^{2+}]_o$ (e.g. Fig. 2), indicate that evoked release is sensitive to stimulation frequency even at very low release rates. This enhancement of evoked release at higher frequencies was progressive, and was accompanied by an increase in the background release rate during the interstimulus interval (see Fig. 6).

Experiments in Co^{2+}

Fig. 4 illustrates the (normalized) relationship between low quantal contents and $[Ca^{2+}]_o$ obtained in four experiments performed in 2–4 mM-Co^{2+}. At the lowest stimulation frequencies (0·5–2 Hz) quantal contents fall slightly above the fourth power prediction (continuous line), but are still described better by a fourth power than by a linear relationship. Quantal contents measured at higher frequencies

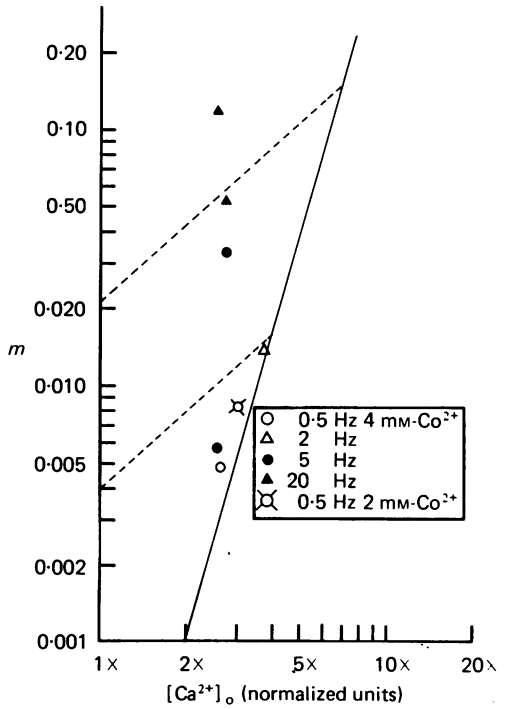

Fig. 4. Double logarithmic plot of average quantal content (m) *vs.* $[Ca^{2+}]_o$ (normalized units), summarizing results obtained in 4 mM-Co^{2+} (2 mM in one case ⌑) at $[Ca^{2+}]_o$ ranging from 0·6 to 1·75 mM). Stimulation frequencies are indicated; note the higher m values at higher frequencies. Average quantal contents were measured and plotted as in Fig. 3. Initial m values (offscale) ranged from 0·20 to 0·53 in 1·9–6 mM-Ca^{2+}. The continuous and dashed lines represent, respectively, fourth power and linear model predictions, as in Fig. 3.

deviate markedly from fourth power predictions. Spontaneous quantal release rates tended to be higher than normal in cobalt (see also Weakly, 1973) and the elevation of the background m.e.p.p. frequency during stimulation was greater and persisted longer after stimulation in 4 mM-Co^{2+} than in 2 mM-Mn^{2+}.

Experiments in Mg^{2+}

Fig. 5 plots the normalized relationship between low m values and $[Ca^{2+}]_o$ found in five experiments performed in 2–10 mM-Mg^{2+}. It can be seen that all quantal contents measured in the presence of Mg^{2+}, even those measured at only 0·5–1 Hz, lie above the fourth power prediction. Higher-than-predicted quantal contents occurred in

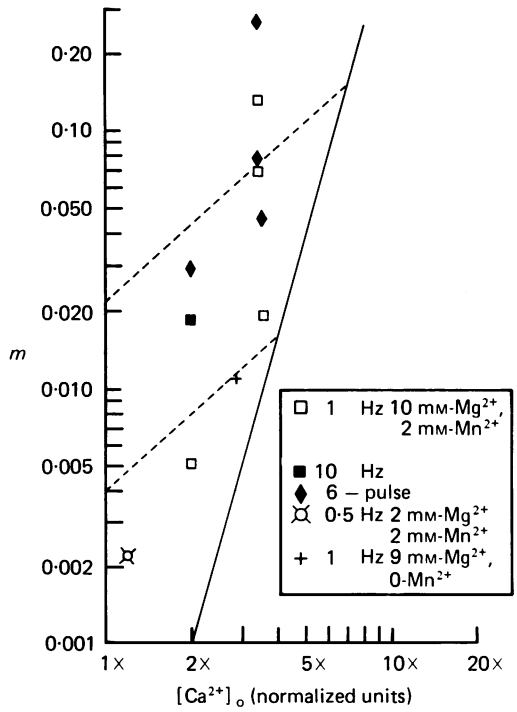

Fig. 5. Double logarithmic plot of average quantal content (m) *vs.* $[Ca^{2+}]_o$ (normalised units), summarizing results obtained in 10 mM-Mg^{2+} plus 2 mM-Mn^{2+} with $[Ca^{2+}]_o$ ranging from 0·3 to 1·3 mM. One preparation was bathed instead in 9 mM-Mg^{2+} alone (+), another in 2 mM-Mg^{2+} plus 2 mM-Mn^{2+} (⌑). Points marked by ◆ represent a stimulus pattern consisting of a cluster of 6 stimuli at 33 Hz applied once each second; the plotted data points were obtained by averaging the m values for all 6 stimuli (see text). Initial m values (offscale) ranged from 0·25 to 1·27 in 0·8–3 mM-Ca^{2+}. Points with the same horizontal co-ordinate were generally obtained from the same junction. Quantal contents were measured and plotted as in Figs. 3 and 4; again, the continuous and dashed lines indicate fourth-power and linear predictions, respectively. Note that all quantal contents lie well above the fourth power prediction, even at 0·5–1 Hz stimulation, and that both 10 Hz stimulation and the clustered six-pulse stimulation increase m above its 1 Hz value.

solutions containing 9 mM-Mg^{2+}, and in solutions containing 2 or 10 mM-Mg^{2+} plus 2 mM-Mn^{2+}. The discrepancy at low frequencies is more marked than that observed in cobalt (compare Figs. 4 and 5). The discrepancy between measured quantal contents and the fourth power prediction increased with increasing stimulus frequency. M.e.p.p. release rates in magnesium were normal (around 1/sec) at rest and during low frequency stimulation (0·5–1 Hz); increasing the stimulation frequency increased background m.e.p.p. release rates, as also observed in Mn and Co.

Effects of stimulus frequency on evoked release at low m

It is well known that increasing the frequency of stimulation increases evoked release rates in neuromuscular junctions where evoked release has been partially

inhibited with Mg. It has been suggested that several processes, with time constants ranging from tens of milliseconds to minutes, contribute to this increase in evoked release (Mallart & Martin, 1967; Magleby, 1973; Magleby & Zengel, 1975, 1976). Results presented in Figs. 2–5 indicate that high frequency stimulation increases evoked release even when control (0·5–2 Hz) evoked release rates are very low. This effect was evident in all solutions tested, but could be demonstrated at lower stimulation frequencies in solutions containing 10 mM-Mg^{2+} than in solutions containing 2 mM-Mn^{2+} alone. Fig. 6 plots the time course of the increase in release rates observed during three episodes of 10 Hz stimulation at a junction bathed in 10 mM-Mg^{2+}, 2 mM-Mn^{2+} and 0·74 mM-Ca^{2+}. Each stimulation episode lasted 100 sec, and was followed by a rest period of several minutes. Each filled circle represents the average release rate (quanta/sec) during the 5 msec evoked release interval in 100 consecutive trials; each open circle represents the corresponding background release rate, measured in the interval 50–97 msec after each stimulus. Note in this Figure that release occurring during the evoked release interval was *not* corrected for the background release rate. There is considerable probabilistic fluctuation, especially in release rates during the evoked release interval, each of which was calculated from only a few (1–12) quanta. However, it can be seen that the 10 Hz stimulation produced a gradual increase in release rates during both the evoked and the background release periods. The increase in background release rate was at least partially reversible, since the background release rate fell during the two rest periods. True evoked release, the *difference* between the release rates measured during evoked and background release intervals, also increased progressively during stimulation.

Several other studies have reported stimulation-induced increases in background m.e.p.p. frequencies under conditions in which evoked release is very low or indetectable (Blioch, Glagoleva, Liberman & Nenashev, 1968; Miledi & Thies, 1971; Hurlbut, Longenecker & Mauro, 1971; Erulkar & Rahamimoff, 1978). With one exception these studies, which used very low $[Ca^{2+}]_o$ (often buffered with EGTA) and 1–10 mM-Mg^{2+}, did not report stimulation-induced increases in *evoked* release. (Miledi & Thies saw a progressive increase in phasic evoked release during 100 Hz stimulation in unbuffered zero Ca^{2+} solutions, which they attributed to a progressive buildup of $[Ca^{2+}]_o$ due to Ca^{2+} leakage from muscle.) Our finding of progressive, stimulation-induced increases in evoked release under very low quantal content conditions may be due to the relatively high $[Ca^{2+}]$ ($\geqslant$ 0·4 mM) in all our solutions, and/or to our sensitive measurement techniques.

In all our solutions the progressive increase in evoked and background release during high frequency stimulation occurred over a fairly long time course ($\geqslant$ 300 sec in Fig. 6), indicating that the frequency enhancement of evoked and background release at low quantal contents has slow component(s). Our data are not sufficient to determine whether this slow increase in evoked release is the same as the potentiation described at higher quantal contents.

We used another paradigm to look for faster components in the frequency enhancement of evoked release at low quantal contents. Preparations in 10 mM-Mg^{2+} plus 2 mM-Mn^{2+} were stimulated with 6-pulse bursts of 33 Hz stimulation delivered at 1 sec intervals. We measured the average evoked release following each of the six

pulses, as well as the average evoked release during conventional 1 Hz stimulation. The 6-pulse stimulation pattern clearly increased the average evoked release rate over the 1 Hz control value (Fig. 5), but we could detect no consistent difference between the average release evoked by the individual pulses within the 6-pulse burst. Release to the sixth pulse did not consistently exceed release to the first pulse,

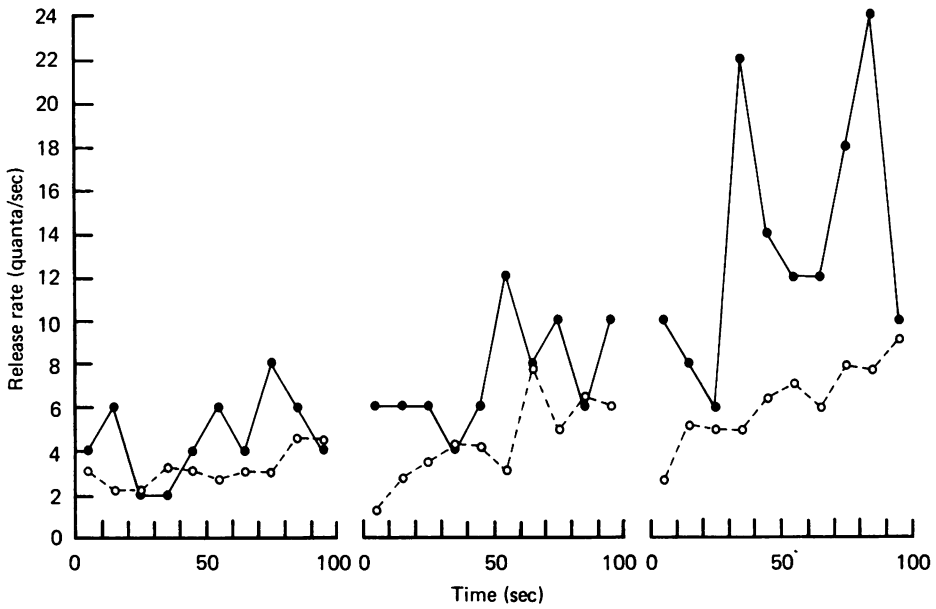

Fig. 6. Increase in evoked and background release rates during 10 Hz stimulation in 0·74 mM-Ca^{2+}, 10 mM-Mg^{2+} and 2 mM-Mn^{2+}. Each point represents the average release rate (quanta/sec) in a particular post-stimulus interval during a series of 100 consecutive stimuli (lasting 10 sec). Filled circles connected by lines represent all quanta released during the evoked release interval, 6–10 msec after each stimulus. Open circles connected by dashed lines represent background release, measured between 50 and 97 msec after each stimulus. True evoked release, the difference between these two curves, increases during the train (delayed release rates, measured 11–40 msec following each stimulus, generally fell between evoked and background release rates). Breaks in the time axis indicate periods of no stimulation, each lasting several minutes. The evoked release rate shows more fluctuation than the background release rate because it was measured from fewer total quanta: the evoked release rate was low, and the evoked release interval (5 msec) was much smaller than the background interval (48 msec).

nor did release to the last 3 pulses consistently exceed release to the first 3 pulses. Analysis of facilitation at higher quantal contents suggests that with this stimulus pattern the average release to the sixth pulse should be at least 2–3 times the average release to the first pulse (Mallart & Martin, 1967; Magleby, 1973). Our failure to detect facilitation might be due simply to the large standard error of our low quantal content measurements. Alternatively, this result might indicate that facilitation is less prominent under the very low quantal content conditions studied here.

DISCUSSION

Fourth power dependence of evoked release on extracellular [Ca^{2+}]

Previous studies have shown that amphibian neuromuscular junctions bathed in solutions containing millimolar concentrations of Mg^{2+}, Co^{2+} or Mn^{2+} show a steep, fourth-power relationship between evoked release and $[Ca^{2+}]_o$ for quantal contents ranging from about 0·5 to 30 (Dodge & Rahamimoff, 1967; Balnave & Gage, 1973; Crawford, 1974). Our study of lower evoked release rates demonstrates that during low frequency (0·5–2 Hz) stimulation in 2 mM-Mn^{2+} (or 4 mM-Co^{2+}) this fourth-power relationship extends down to quantal contents as low as about 0·002, where evoked release rates approach the spontaneous, or 'background', rate of quantal release. Thus under these conditions a fourth power model can describe the relationship between evoked release and $[Ca^{2+}]_o$ over four orders of magnitude of evoked release.

This conclusion differs from that of Crawford (1974), who reported that the relationship between evoked release and $[Ca^{2+}]_o$ becomes linear at very low quantal contents. Crawford used either 4 mM-Co^{2+} or a combination of Mg^{2+} plus a Ca^{2+} buffer (EDTA or citrate) to reduce Ca^{2+}-evoked release, and he stimulated faster (up to 10 Hz) the lower the quantal content. We also observed quantal contents higher than fourth power predictions in 2–10 mM-Mg^{2+} (see section on Mg^{2+} below), and we found that stimulation at 5–10 Hz produced higher-than-predicted quantal contents in all solutions. However, the fact that at lower frequencies ($\leqslant$ 2 Hz in 2 mM-Mn^{2+} or 4 mM-Co^{2+}) the relationship between evoked release and $[Ca^{2+}]_o$ remains very close to fourth power down to very low quantal contents, leads us to conclude that in this preparation the basic relationship between $[Ca^{2+}]_o$ and evoked release is fourth order. The higher-than-fourth-power quantal contents that we and Crawford observed at stimulation frequencies exceeding 5 Hz are probably due to some of the same frequency-dependent processes (e.g. augmentation, potentiation) that have been shown to increase evoked release at somewhat higher quantal contents (Magleby & Zengel, 1975, 1976).

There are several possible explanations for the fourth-power relationship between evoked release and extracellular [Ca^{2+}]. Perhaps the simplest explanation is that four calcium ions are necessary to activate a release site. This activation could be achieved by a cluster of four identical Ca-receptor complexes (Dodge & Rahamimoff, 1967), by four calcium ions binding to a single receptor molecule (Hubbard, Jones & Landau, 1968*b*), or by reactions involving multiple Ca-binding sites (say X, Y, Z), with evoked release proportional to, for example, the product $[CaX][Ca_2Y][CaZ]$. Several of the Ca-dependent regulatory proteins (e.g. calmodulin, troponin C) extracted from muscle, brain and other tissues have four Ca-binding sites (reviewed in Cheung, 1980). Some proposed models of facilitation have used third or fourth power schemes (Rahamimoff, 1968; Barrett & Stevens, 1972*b*; Younkin, 1974; Zengel & Magleby, 1977), and multiple site, multiplicative models have been proposed to explain some aspects of facilitation (Balnave & Gage, 1974) and the relationships between facilitation, augmentation and potentiation (Magleby, 1973; Landau, Smolinsky & Lass, 1973; Zengel & Magleby, 1977). Another possible expla-

nation for the nonlinear relationship between evoked release and extracellular [Ca^{2+}] is that release is exponentially (rather than linearly) related to the number of Ca-activated sites in the nerve terminal (Cooke, Okamoto & Quastel, 1973). Present data are not sufficient to decide which (if any) of these proposed explanations are correct. One constraint on these models is that at least some of the Ca-requiring steps involved in evoked release must occur intracellularly, since it has been shown that Ca must enter the nerve terminal to accelerate release (Katz & Miledi, 1967, 1969, 1977; Miledi, 1973; Llinas & Nicholson, 1975). The nonlinear dependence of evoked release on extracellular [Ca^{2+}] could arise during or after Ca entry. Our data (Figs. 2, 3*A*, 4) impose the additional constraint that the relationship between evoked release and $[Ca^{2+}]_o$ remains steeply nonlinear down to the lowest measurable levels of evoked release.

Possible mechanisms for deviations from fourth power in Mg^{2+}

At the lowest evoked release rates studied here, all quantal contents measured in solutions containing Mg^{2+} exceed fourth power predictions, even at low stimulation frequencies (Fig. 5). Furthermore, careful examination of the low frequency data collected in 2 mM-Mn^{2+} (Fig. 3*A*) and in 2–4 mM-Co^{2+} (Fig. 4) indicates that even in the absence of Mg^{2+} there is a tendency for low quantal contents to fall slightly above the fourth power line. One possible explanation for these deviations comes from a model developed by Jenkinson (1957) and Dodge & Rahamimoff (1967) to describe the relationship between extracellular [Ca^{2+}] and [Mg^{2+}] and evoked release. This model assumes that one step in evoked release requires the combination of Ca^{2+} with a receptor site X, and that Mg^{2+} (or Mn^{2+} or Co^{2+}) inhibits release by competing with Ca^{2+} for this receptor site (see Balnave & Gage, 1973 for Mn^{2+}; Weakly, 1973 and Crawford, 1974 for Co^{2+}). Recent evidence suggests that the site of this competition is mainly extracellular, perhaps at a site controlling access to the voltage-dependent Ca^{2+} channels, since Mg^{2+}, Mn^{2+} and Co^{2+} all inhibit Ca^{2+} entry into stimulated nerve and muscle (Baker *et al.* 1973; Hagiwara, Fukuda & Eaton, 1974). Assuming that sites occupied by Mg^{2+}, Mn^{2+} or Co^{2+} do not contribute to evoked release, and combining equations for Mg^{2+}, Mn^{2+} and Co^{2+} given in the papers cited above, evoked release (R_e) would be described by

$$R_e = A\left(\frac{[Ca^{2+}]_o}{1+\frac{[Ca^{2+}]_o}{K_{Ca}}+\frac{[Mg^{2+}]_o}{K_{Mg}}+\frac{[Mn^{2+}]_o}{K_{Mn}}+\frac{[Co^{2+}]_o}{K_{Co}}}\right)^n \qquad (3)$$

where A is a proportionality constant, n is integral (4 in the fourth power model) and K_{ion} represents the dissociation constant measured for the particular ion. According to the above-cited papers, K_{Ca} = 1·1–1·5 mM, K_{Mg} = 3–4 mM, K_{Mn} = 0·15 mM, and K_{Co} = 0·07–0·18 mM. As $[Ca^{2+}]_o$ is reduced, the denominator of this expression decreases, thus reducing the slope of the log release *vs* log $[Ca^{2+}]_o$ curve at low $[Ca^{2+}]_o$. Therefore this model predicts that quantal contents measured at low $[Ca^{2+}]_o$ will be slightly greater than the quantal contents predicted from a fourth power line extrapolated from quantal contents measured at higher $[Ca^{2+}]_o$. We calculated the magnitude of the deviation predicted by this model by inserting into eqn. (3)

the divalent ion concentrations and dissociation constants appropriate to each low frequency (0·5–2 Hz) quantal content plotted in Figs. 3–5. For the seven low-frequency quantal contents measured in 2 mM-Mn^{2+} (Fig. 3*A*) this model predicts that quantal contents should be (on average) 26 % higher (range 21–32 %) than values predicted from a fourth power line; the average measured discrepancy was 38 % (range −50 to +186 %). Predicted discrepancies in 2–4 mM-Co^{2+} (Fig. 4) averaged 29 % (range 15–46 % for three junctions), compared to a measured mean discrepancy of 42 % (range 8–60 %). Thus the model of eqn. (3) (with $n = 4$) can account for much of the deviation from the fourth power line measured in Mn^{2+} and Co^{2+}. (A more sophisticated statistical comparison of predicted and measured values is difficult because of the small sample sizes and the large variance of the discrepancies measured in Mn^{2+}.)

In contrast, the model of eqn. (3) can account for relatively little of the deviation from the fourth power line measured in the presence of Mg^{2+} (Fig. 5). Here the measured deviations averaged 460 % (range 46–1600 % for five junctions), while the predicted mean deviation for the same data was only 43 % (range 32–59 %). Thus it seems that Mg^{2+}, which probably enters the stimulated nerve terminal (Baker & Crawford, 1972), somehow increases evoked release under very low quantal content conditions. This entering Mg^{2+} might contribute to evoked release either directly, by weakly activating release sites, or indirectly, by displacing Ca^{2+} from various intracellular binding sites. The resulting elevated intracellular [Ca^{2+}], summing with the Ca^{2+} that enters the nerve terminal during the action potential, could cause measured evoked quantal contents to exceed fourth power predictions, especially at low quantal contents (see eqn. (5) below). Other studies also suggest that Mg^{2+} can contribute to release when Ca^{2+}-activated release is absent or markedly reduced. For example, in Ca^{2+}-deficient solutions post-tetanic m.e.p.p. frequencies increase with increasing $[Mg^{2+}]_o$ (Blioch *et al.* 1968; Hurlbut *et al.* 1971), and Mg^{2+} can substitute for Ca^{2+} in supporting the massive quantal release seen in neuromuscular preparations treated with black widow spider venom (Ornberg, 1977; Gorio & Mauro, 1979; Misler & Hurlbut, 1979). Additional evidence for Mg^{2+}-dependent release is reviewed in van der Kloot (1978).

The mechanisms proposed to explain deviations from fourth power in Mg^{2+} might also contribute to the slightly higher-than-predicted quantal contents seen at low stimulation frequencies in Co^{2+} and Mn^{2+}. Like Mg^{2+}, Co^{2+} and Mn^{2+} can support quantal release in the presence of black widow spider venom (Gorio & Mauro, 1979; Misler & Hurlbut, 1979). Also, Kita & van der Kloot (1973) found that in the absence of Ca^{2+}, Co^{2+} enhanced resting, K^+-stimulated, and post-tetanic m.e.p.p. frequencies. Perhaps Co^{2+} and Mn^{2+} produce smaller deviations from fourth power than Mg^{2+} because they enter the stimulated nerve terminal less readily than Mg^{2+}, and thus have less access to intracellular release sites.

Spontaneous quantal release and intracellular [Ca^{2+}]

Several indirect arguments suggest that spontaneous quantal release from resting (non-depolarized) nerve terminals does not have the same dependence on intracellular [Ca^{2+}] as evoked release. First, resting quantal release is much less dependent

on extracellular [Ca^{2+}] than evoked release (del Castillo & Katz, 1954; Hubbard, Jones & Landau, 1968*a*; Miledi & Thies, 1971; Erulkar, Rahamimoff & Rotshenker, 1978). One possible explanation for this finding is that there is a Ca-independent fraction of spontaneous release (see also Quastel, Hackett & Cooke, 1971). Second, spontaneous quantal release from resting frog motor nerve terminals has a much steeper temperature dependence (temperature range 10–20 °C) than the $[Ca^{2+}]_o$-dependent release evoked by nerve stimulation or depolarization with high extracellular [K^+] (Barrett, Barnett, Botz, Chang & Mahaffey, 1978). A third argument is based on our finding that the relationship between evoked release and $[Ca^{2+}]_o$ remains fourth power down to very low evoked release rates (Figs. 2, 3*A*). If evoked and resting release both depend on *intra*cellular [Ca^{2+}], then one would instead expect that the relationship between evoked release and *extra*cellular [Ca^{2+}] would deviate from fourth power (by becoming less steep) as $[Ca^{2+}]_o$ is reduced. To predict the extent of the deviation expected for a fourth power model of spontaneous and evoked release, we derived the following equations (see also Barrett & Stevens, 1972*b*; Crawford, 1974; Younkin, 1974):

We assume that both evoked and spontaneous release rates are proportional to the fourth power of [Ca^{2+}] in a common intracellular compartment, and that the Ca influx accompanying each action potential contributes to this Ca^{2+} pool by an amount proportional to $[Ca^{2+}]_o$, such that:

$$R_s = K[Ca]_r^4 \tag{4}$$

$$R_e = K(\alpha[Ca]_o + [Ca]_r)^4 - R_s, \tag{5}$$

where R_s and R_e are spontaneous (background) and evoked release rates, respectively, K and α are proportionality constants, $[Ca]_r$ is the [Ca^{2+}] in the resting terminal before the action potential, and $\alpha[Ca]_o$ is the increment in intracellular [Ca^{2+}] contributed by the action potential. These equations can be simplified to:

$$\sqrt[4]{(R_e + R_s)} = \beta[Ca]_o + \sqrt[4]{(R_s}, \tag{6}$$

where $\beta = \alpha(\sqrt[4]{K})$. β can be calculated from measured values of R_e and R_s at one $[Ca^{2+}]_o$, and this value of β, plus the measured value of R_s at a second $[Ca^{2+}]_o$, can be used to predict R_e at the second $[Ca^{2+}]_o$.

This model can be tested using data like those of Figs. 2 and 3*A*, where the lowest measured rates of evoked release approached the spontaneous release rates. The dashed line in Fig. 7 shows the predicted relationship between evoked release and $[Ca^{2+}]_o$ for the data of Fig. 2 using this 'common Ca^{2+} pool, fourth power' model. The evoked release rate predicted for 0·8 mM-Ca^{2+}_0 is 6·7/sec, far greater than the evoked release rate of 1·2/sec actually measured at this $[Ca^{2+}]_o$. Even if we assume that only 10 % of the resting spontaneous release rate has the Ca^{2+} dependence of eqn. (4), the predicted evoked release rate is still three times the observed evoked rate. We also computed the evoked release rate expected if spontaneous release has a *linear*, and evoked release a fourth power, dependence on intracellular $[Ca^{2+}]_o$ (not shown). Even with the most favourable choice of parameter values, the evoked release rate predicted using this latter model also far exceeded that actually measured, even if only 10 % of the spontaneous release rate was assumed to be Ca-dependent. Thus our models assuming that spontaneous quantal release results from a linear or a fourth power dependence on intracellular [Ca^{2+}] do not accurately predict the low quantal

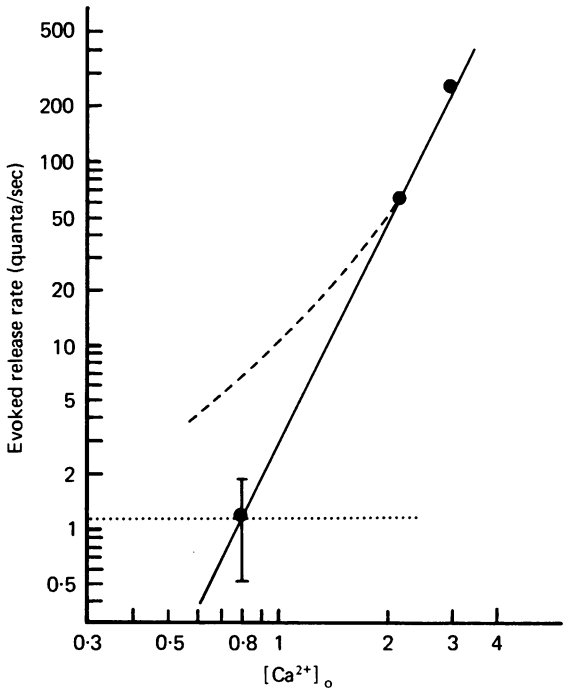

Fig. 7. Predicted double logarithmic relationships between evoked release (quanta/sec) and $[Ca^{2+}]_o$ for the data of Fig. 2. Circles indicate data recorded at 2 Hz in 2 mM-Mn^{2+} (points 1, 2 and 6 of Fig. 2, with quantal contents converted to evoked release rates by dividing by the evoked release interval, 3 msec). The dashed line, calculated using eqn. (6) in the text, represents the relationship predicted if both spontaneous and evoked release have a similar fourth-power dependence on $[Ca^{2+}]$ in a common intracellular pool (the parameter β was calculated using data measured in 2·2 mM-Ca: evoked quantal content 0·19 or 63·3 quanta/sec, spontaneous release rate 1·26/sec). The continuous line, drawn with a slope of 4 through the data point at 2·2 mM-Ca, represents the evoked release predicted assuming that only evoked release has a fourth power dependence on intracellular $[Ca^{2+}]$. This latter model predicts the evoked release rate measured in 0·8 mM-Ca^{2+} (m = 0·0036, or 1·2 quanta/sec) far better than the common fourth-power model of eqn. (6). The dotted line indicates the spontaneous release rate measured in 0·8 mM-Ca, 1·14/sec.

content data of Fig. 2. These data, and indeed all data collected at low frequencies in 2 mM-Mn^{2+} or 4 mM-Co^{2+}, are much better fitted by a fourth power relationship between evoked release and extracellular $[Ca^{2+}]$ (continuous lines, Figs. 2, 3*A*, 4, 7, perhaps as modified by eqn. (3)), with no contribution from 'resting' intracellular $[Ca^{2+}]$. This result may mean that under these experimental conditions most of the spontaneous release from resting (non-depolarized) nerve terminals is not Ca^{2+}-dependent, or that evoked and spontaneous release do not share the same intracellular Ca^{2+} pool. We favour the former interpretation because of the very different temperature sensitivities of resting and Ca^{2+}-dependent release (Barrett *et al.* 1978).

We therefore suggest that the spontaneous quantal release (as distinct from evoked and delayed release) from frog motor nerve terminals has at least two components,

a Ca-independent component which constitutes a large fraction of the spontaneous release from nondepolarized or lightly ($\leqslant$ 2 Hz) stimulated terminals, and a Ca-dependent component which dominates the background release recorded from depolarized or rapidly stimulated terminals. Given a Ca-independent component of spontaneous quantal release, the rate of resting release may not always reflect the level of intracellular [Ca^{2+}], and changes in m.e.p.p. frequency may not always imply a corresponding change in intracellular [Ca^{2+}].

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