J. Physiol. (1974), **240**, *pp.* 255–278
With 10 *text-figures*
Printed in Great Britain

THE DEPENDENCE OF EVOKED TRANSMITTER RELEASE ON EXTERNAL CALCIUM IONS AT VERY LOW MEAN QUANTAL CONTENTS

By A. C. CRAWFORD

From the Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG

(*Received 25 October 1973*)

SUMMARY

1. The mean quantal content of the frog end-plate potential was examined under conditions that reduced evoked transmitter release to very low values.

2. When the calcium concentration of the Ringer is reduced below 10^{-4} M a deviation occurs from the fourth power dependence of the mean quantal content (m) on the calcium concentration such that more quanta are released than is expected from the behaviour of m at higher calcium concentrations.

3. At 10^{-5} M this extra quantal release is more than two orders of magnitude greater than that predicted by the fourth power relationship.

4. The calcium dependence of very low values of m was studied in low calcium Ringer in which the calcium was buffered by either citrate or EDTA. It was found that in the fourth power dependence of m on the external calcium concentration changes rather suddenly to an approximately linear dependence.

5. The inclusion of small concentrations of cobalt in the Ringer was found to reduce m to very low values even in the presence of millimolar concentrations of calcium.

6. The fourth power dependence of m on the external calcium concentration at high values of m was unaffected by the presence of cobalt. At low quantal contents the transition to a linear dependence on external calcium was again seen, but was shifted to calcium concentrations that did not require buffering.

7. The fourth power to linear transition is discussed in terms of its relevance to the relationship between m and the m.e.p.p. frequency.

INTRODUCTION

It is now established that calcium ions play a crucial role in linking the invasion of a nerve terminal by an action potential to the release of transmitter. While the initial step in this process appears to be an entry of calcium into the terminal (Katz & Miledi, 1967*a*, *b*, 1969) subsequent events are poorly understood. Much work has been devoted recently to understanding precisely how external calcium and magnesium interact to determine the mean quantal content of the end-plate potential (*m*) (Jenkinson, 1957; Hubbard, Jones & Landau, 1968*a*, *b*; Cooke, Okamoto & Quastel, 1973; Dodge & Rahamimoff, 1967). This interaction is well described by assuming that external calcium and magnesium compete for an activator molecule and that the release of a quantum of transmitter requires the co-operative action of four calcium-activated complexes.

While the site of action of calcium in the release process is not definitely established it seems likely, in view of the fact that calcium entry is obligatory for evoked release of transmitter, that calcium acts at a site located inside the presynaptic terminal. The simple Michaelis–Menton kinetics that describe so well the interaction of external calcium and magnesium in the release of transmitter are however most directly applicable to a site of action of calcium located on the outer surface of the membrane. The competitive kinetics of external divalent cation action can easily be transferred to an internal site if one assumes, as do Katz & Miledi (1969) and Miledi & Thies (1971), that entry of calcium during the presynaptic action potential causes a transient rise in the internal concentration of calcium that is linearly related to the external calcium concentration. Provided that the calcium influx is linearly related to the external calcium concentration (and this is the behaviour of the extra entry of calcium per impulse in squid giant axons found by Hodgkin & Keynes, 1957) and that the ensuing rise in internal calcium is fast compared to any sequestering processes in the terminal, this assumption may be valid.

The role of calcium in the spontaneous release of transmitter is rather obscure. External calcium is mainly effective in elevating the miniature end-plate potential (m.e.p.p.) frequency under conditions when the resting calcium leak into the terminals is presumed to be high but in normal Ringer spontaneous release is largely insensitive to this ion. A variety of other conditions thought to raise the resting calcium inside the terminals also raise the m.e.p.p. frequency (summarized by Baker, 1972). This and the observation of Miledi (1973), that intracellular iontophoresis of calcium into the giant presynaptic terminals of the squid stellate ganglion raises the miniature post-synaptic potential frequency, make it attractive to

suppose that the rate of spontaneous transmitter release is partly determined by the resting intracellular calcium concentration.

It is far from clear whether spontaneous and evoked release of transmitter require the binding of calcium to the same site with the same kinetics. This paper attempts to examine the kinetics of evoked transmitter release under two sets of conditions when the mean quantal content and presumably the calcium entry during an action potential, are very low. This situation has been achieved by either reducing the external concentration of calcium to very low values, or by adding a strong competitive inhibitor of calcium entry in nerve to the Ringer. Under these conditions when the evoked release rate approaches the spontaneous release rate (Miledi & Thies, 1971) the changes in internal calcium concentration resulting from entry may approach the resting internal calcium concentration and yield information relevant to an interpretation of the calcium kinetics of spontaneous release. The results show a marked change in the calcium dependence of m when m is very small and can tentatively be interpreted as indicating a change in the kinetics of the action of calcium.

Some of these results have been the subject of a brief communication (Crawford, 1973).

METHODS

Studies on transmitter release were carried out on frog (*Rana temporaria*) sartorius-sciatic preparations using standard intracellular recording techniques. All experiments were performed at room temperature, the sciatic nerve being stimulated with a suction electrode. Micro-electrodes were filled with 3 M-KCl and had resistances of 10–15 MΩ. The Perspex chamber in which the preparation was fixed had a volume of about 5 ml. and solutions were changed by flowing 50–100 ml. through the bath.

Solutions. The compositions of the main solutions are given in Table 1. A high concentration of Tris buffer was included in all solutions because of the extreme pH sensitivity of the calcium buffers used. The pH was adjusted to 7·2 with NaOH and all solutions had a nominal tonicity of 243·8 m-osmole/l.

Calcium buffers. Two calcium chelating agents, citrate and EDTA, were used to control the ionized (free) calcium concentration in the range 10^{-5}–10^{-4} M. Fig. 1 shows the buffer curves for *A*, EDTA and *B*, citrate at pH 7·2 and at the concentrations given in the legend. For any free calcium concentration the steepness of the slope of the graph indicates the buffering power in that range. Calcium concentrations above 10^{-4} mM were not buffered. At calcium concentrations below about 5×10^{-6} M the mean quantal content of the end-plate potential (m), became too small to be measured by the techniques employed here. As can be seen from Fig. 1 neither buffer controls the free calcium concentration adequately over the whole of the range 10^{-5}–10^{-4} M. The higher concentrations fall well within the citrate range and the lower part of the range is adequately buffered by EDTA. The main challenge to these buffering systems is the leak of calcium out of the muscle (Miledi & Thies, 1971). Since Shanes & Bianchi (1959) found that more than half the muscle calcium was lost per hour Ringer solutions were never left unchanged on the preparation for more than 30 min.

Free calcium estimations. Samples of the Ringer surrounding the preparation were

TABLE 1. Composition of solutions (mM)

Ion	*A*	*B*	*C*	*D*	*E*	*F*	*G*
Na	114·5	112·4	104·9	94·9	109·9	88·9	113·4
K	2·0	2·0	2·0	2·0	2·0	2·0	2·0
Ca	1·8	—	5·0	10·0	—	15·0	—
Mg	1·8	5·0	5·0	10·0	10·0	—	—
Co	—	—	—	—	—	4·0	4·0
HCO_3	2·5	—	—	—	—	—	—
Cl	123·7	114·4	116·9	111·9	106·4	131·4	125·9
Citrate	—	5·0	5·0	—	—	—	—
EDTA	—	—	—	10·0	10·0	—	—
Tris-Cl	—	5·0	5·0	5·0	5·0	2·5	2·5

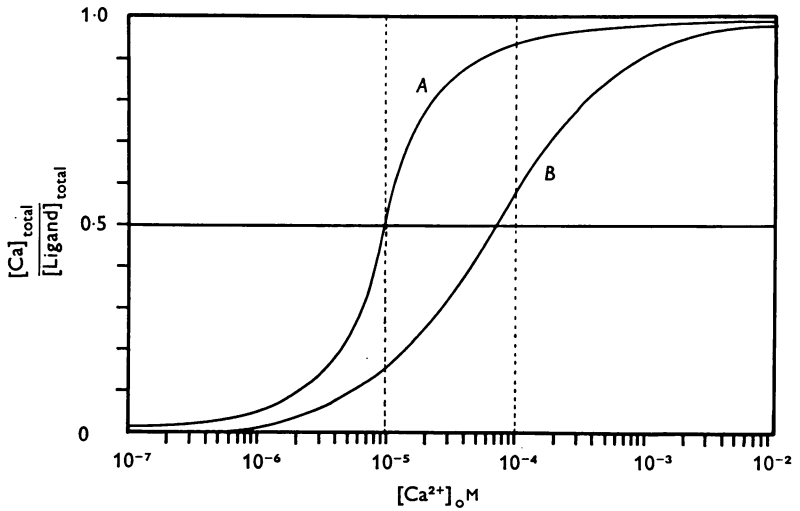

Fig. 1. Buffer curves for citrate and EDTA buffered Ringer at pH 7·2. Ordinate: ratio of the total concentration of calcium to the total concentration of ligand ($[Ca]_{total}/[Ligand]_{total}$). Abscissa: ionized calcium concentration (M). Curve *A*: 10 mM-EDTA and 10 mM magnesium present throughout. Curve *B*: 5 mM citrate and 5 mM magnesium in all solutions. Both curves at 20° C, using the stability constants of Sillen & Martell (1964).

routinely taken and the ionized calcium estimated using the change in absorption between 470 and 542 nm of the calcium indicator Murexide (Koch-light) (Ohnishi & Ebashi, 1963). Nominally 'calcium-free' Ringer (i.e. a Ringer to which no calcium had been added) was found to contain about 10^{-5} M calcium. In Ringer buffered with respect to calcium the free calcium agreed well with that calculated from the known stability constants (Sillen & Martell, 1964; Portzehl, Caldwell & Rüegg, 1964). No significant change occurred in the free calcium concentration of buffered Ringer over a period of an hour but the bath calcium at least doubled if the preparation was left for 6 hr.

Estimation of the mean quantal content (*m*). The quantal contents of successive

end-plate potentials in a long series of trials has been shown to be described by a Poisson distribution provided the mean quantal content is low (del Castillo & Katz, 1954*a*). The method of estimating the standard error of the mean of a Poisson distribution depends on the product of m and the total number of trials (N). For $Nm > 30$ the approximation of a Poisson distribution to normality can be used, the S.E. of mean being given (Martin, 1966) by

$$\text{S.E. of mean} = \left(\frac{m}{N}\right)^{\frac{1}{2}}.$$

In those experiments with low calcium Ringer the value of m fell as low as 3×10^{-3}, i.e. 3 quanta released on average per 1000 trials. Very large numbers of trials (up to 10^4) were then carried out to reduce the S.E. of mean of a single estimation of m. All measurements were rejected if the S.E. of mean was greater than 25 %. In the few cases where the product of N and m was less than 30 the S.E. of mean was calculated from full confidence limits for a Poisson distribution (Pearson & Hartley, 1966).

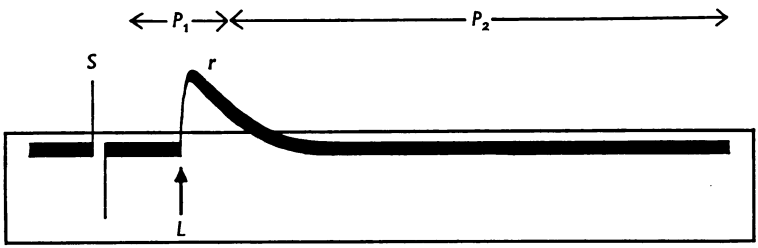

Fig. 2. Diagram of the masking method of measuring low mean quantal contents. The heavy line represents the oscilloscope trace (duration 50 msec) with stimulus artifact, S, and unit quantal release, r. The box shows the limit of the mask and is adjusted to display only part of the stimulus artifact and any quantal releases above it. L is the mean latency of the end-plate potential and period P_1 is ± 3 S.D. of the mean latency. P_1 proved to be about 5 msec at room temperature. Period P_2 lasts from the end of P_1 to the end of the trace, this being used to estimate the intra-tetanic miniature end-plate potential frequency. P_2 was normally about 35–40 msec.

All estimates of m were derived from photographs of the oscilloscope display of the electrical recordings. Since for the very low values of m the majority of trials are failures, the oscilloscope trace was masked as in Fig. 2 in such a way that only the stimulus artifact and quantal releases, whether spontaneous or evoked, rose above the mask and were photographed. Failures were not recorded. This method greatly reduced the amount of film used and facilitated the analysis of the records. Fig. 3 shows a sample record using the masking technique from an end-plate in two calcium solutions. Masked photographs were only used when m was less than 10^{-2} and required a large mean miniature end-plate potential (m.e.p.p.) amplitude to ensure all unit releases were detected above the mask. In all experiments presented here the amplitude histogram of unmasked spontaneous quanta was compared with that of the masked quantal releases, both being measured in the lowest calcium-Ringer of that experiment where quantal amplitudes were smallest. Measurements where masking hid more than 10 % of the m.e.p.p, population were rejected.

The main difficulty in analysing the records was to distinguish evoked release from spontaneous release of transmitter. The procedure adopted was as follows and is similar to that used by Miledi & Thies (1971). The mean latency of the e.p.p. and its S.D. were measured in a Ringer that gave quantal release nearly every trial. A period (P_1 in Fig. 2) was then set after the stimulus artifact that centred on the mean latency (L) and extended for ± 3 S.D. from this point. It was assumed that all evoked releases fell within P_1 in any calcium solution. Since this period also contains m.e.p.p.s indistinguishable from unit evoked releases it was necessary to make a correction for these. The mean frequency of m.e.p.p.s within the tetanus was derived from P_2 in Fig. 2, the remainder of the oscilloscope trace following P_1. Then,

$$n_e = n_{P_1} - n_{P_2} \cdot \frac{P_1}{P_2},$$

where n_e is the number of evoked releases in the trial, n_{P_1} and n_{P_2} are the total numbers of quanta falling within P_1 and P_2 respectively, and P_1 and P_2 are the durations of these periods. The value of m can then be derived in two ways.

(i) *Directly* if all releases are unit potentials since $m_1 = (n_e/N)$. Provided that m is small (less than 10^{-2}) and that a Poisson distribution holds, the assumption that all evoked releases are single quanta is a reasonable approximation. With $m = 10^{-2}$ the probability of a release with a quantal content of 2 is only $4{\cdot}95 \times 10^{-5}$. Since multiple releases are not ignored but counted as single quanta anyway, the error involved in assuming unit release is very much less than the sampling error.

(ii) *Failures.* The mean quantal content is given by $m_0 = \ln (N/N_0)$ where N is the total number of trials and N_0 the number of failures (del Castillo & Katz, 1954*b*).

Stimulation rates. Preparations are not stationary with respect to m indefinitely. In order to carry out several estimations of m involving large numbers of trials on the same junction, it was necessary to raise the normal trial repetition rate from 1 Hz (Dodge & Rahamimoff, 1967) to up to 10 Hz. Even in very low calcium Ringer Miledi & Thies (1971) found that rapid tetanization increased the m.e.p.p. frequency. However, potentiation within the tetanus of the m.e.p.p. frequency is known to be reduced as the external calcium concentration falls (Miledi & Thies, 1971). The stimulation rate actually used was chosen so that no facilitatory effects were seen; the higher the stimulation rate the lower the calcium concentration. Facilitatory effects were detected by either (*a*) dividing the summed P_2 into equal non-overlapping periods and estimating the intratetanic m.e.p.p. frequency at successive periods of the tetanus, or (*b*) for the larger values of m ($> 10^{-2}$) the trial was divided in a similar way and any increase in m with time noted. It was not possible to test for a drift in m when the total number of evoked responses was small. But since increases in m due to repetitive stimulation are always accompanied by increases in m.e.p.p. frequency (f) (del Castillo & Katz, 1954*a*; Brooks, 1956; Hubbard, 1963; Miledi & Thies, 1971; Rahamimoff & Yaari, 1973) the absence of any change in f was taken as evidence that m had not increased even when the stability of m during a tetanus could not be checked directly. Any measurements that showed facilitation were rejected.

These experiments depend on the assumption that the system is stationary over the period of the experiment. A rough check that proved very useful was to begin and end the experiment in the same solution, usually 0·6 mM calcium Ringer, where the sampling error was small. All experiments were rejected if initial and final values of m disagreed by more than 20 %. A progressive fall in m during the experiment proved to be a major source of error. In seventeen experiments using EDTA buffers only 8 satisfied the above criterion for a stationary end-plate. It is possible

that the reduction in the value of m was caused by progressive depolarization of the terminals during the long periods in low calcium Ringer (Frankenhaeuser, 1957).

RESULTS

Experiments in unbuffered Ringer

Previous studies on the relationship between evoked transmitter release and the external calcium concentration ($[Ca]_o$) (Jenkinson, 1957; Dodge & Rahamimoff, 1967; Hubbard *et al.* 1968*a*; Cooke *et al.* 1973) have not pursued the $[Ca]_o$ below 10^{-4} M, since in simple unbuffered Ringer problems arise not only with calcium contamination from impurities in the other

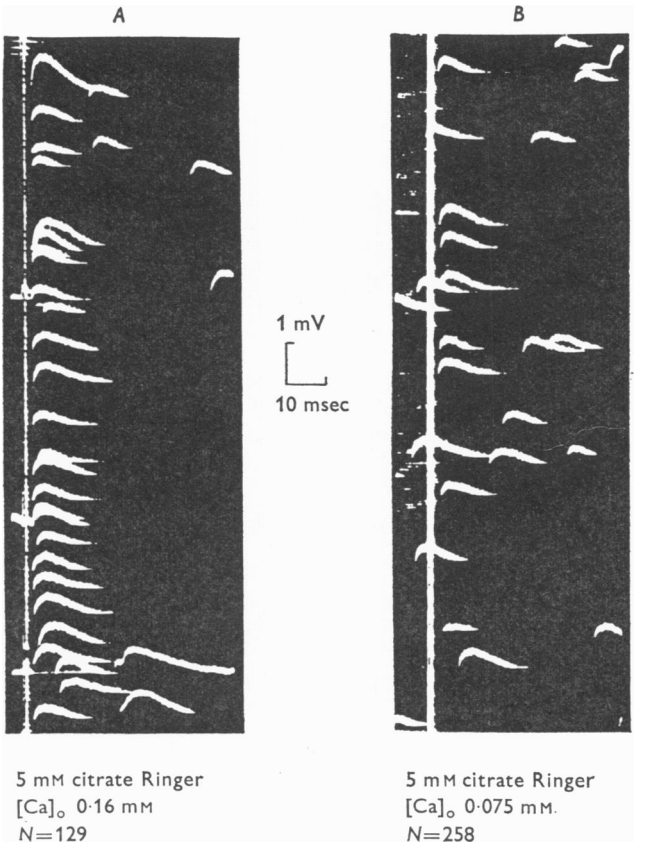

Fig. 3. Sample records of transmitter release during two tetani in two different low calcium concentrations. Both photographs illustrate the masking technique. *A*, 5 mM citrate-buffered Ringer at a free calcium concentration of 0·16 mM. *B*, 5 mM citrate-buffered Ringer at a free calcium concentration of 0·075 mM. Both solutions contain 5 mM total magnesium. Scales are 1 mV and 10 msec. Temperature 20·5° C. N is the total number of trials in each photograph.

salts of the Ringer (Curtis, 1963; Miledi & Thies, 1971), but also with calcium leakage from the preparation (Shanes & Bianchi, 1959; Miledi & Thies, 1971). While it is not easy to avoid these sources of contamination the extent of the changes in calcium concentration of the bathing solution can be measured. Nine experiments, of which Fig. 4 illustrates an example, were carried out in which the progressive omission of calcium

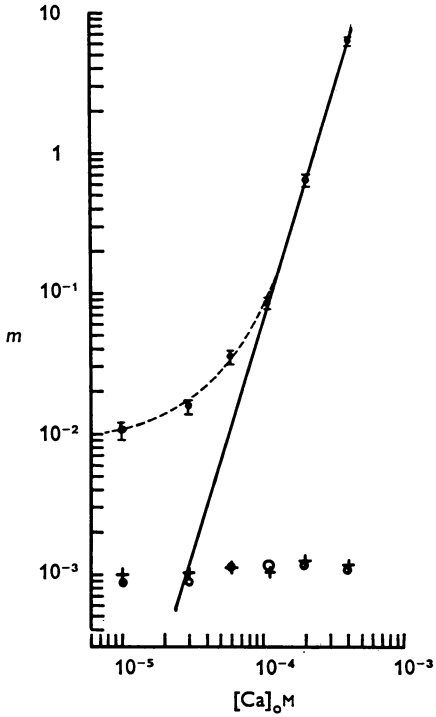

Fig. 4. Double logarithmic plot of the dependence of transmitter release on the external calcium concentration in Ringer unbuffered with respect to calcium. Abscissa; the free calcium concentration (M) as determined by murexide assay on bath samples of the Ringer (solution *A* in Table 1 with $CaCl_2$ replaced isotonically by NaCl). Ordinate: ● the mean quantal content of the end-plate potential (± S.E.); ○ m.e.p.p. frequency between periods of stimulation and + m.e.p.p. frequency within the tetanus. M.e.p.p. frequencies are expressed in quanta/msec. Temperature 23° C.

from the Ringer lowered the $[Ca]_o$ to about 10^{-5} M. In all solutions the concentration of magnesium was raised to 2 mM to reduce the likelihood of action potential conduction failure (Dodge & Rahamimoff, 1967) and calcium chloride replaced isotonically by sodium chloride. Samples of the Ringer surrounding the preparation showed that nominally zero-calcium Ringer in fact contained about 10^{-5} M free calcium. Fig. 4 is a plot of the

mean quantal content of the end-plate potential (m) against the Murexide assayed free calcium concentration, both on logarithmic co-ordinates. At a $[Ca]_o$ above 10^{-4} M the curve is a straight line with a slope in the nine experiments that fell in the range 3·5–4·0, confirming the finding of Dodge & Rahamimoff (1967). Below a $[Ca]_o$ of 10^{-4} M the points deviate smoothly from the fourth power line, there being more quanta released at low $[Ca]_o$ than is expected from the behaviour of release when the $[Ca]_o$ is above 10^{-4} M. At a $[Ca]_o$ of 10^{-5} M this extra release amounts to more than two orders of magnitude more quanta than expected. The mean quantal content at a $[Ca]_o$ of 10^{-5} M averaged $(7{\cdot}2 \pm 3{\cdot}1$ S.E.$) \times 10^{-2}$ in nine experiments whereas extrapolating the fourth power curve as in Fig. 4 predicts values of m at this $[Ca]_o$ of less than 10^{-4}.

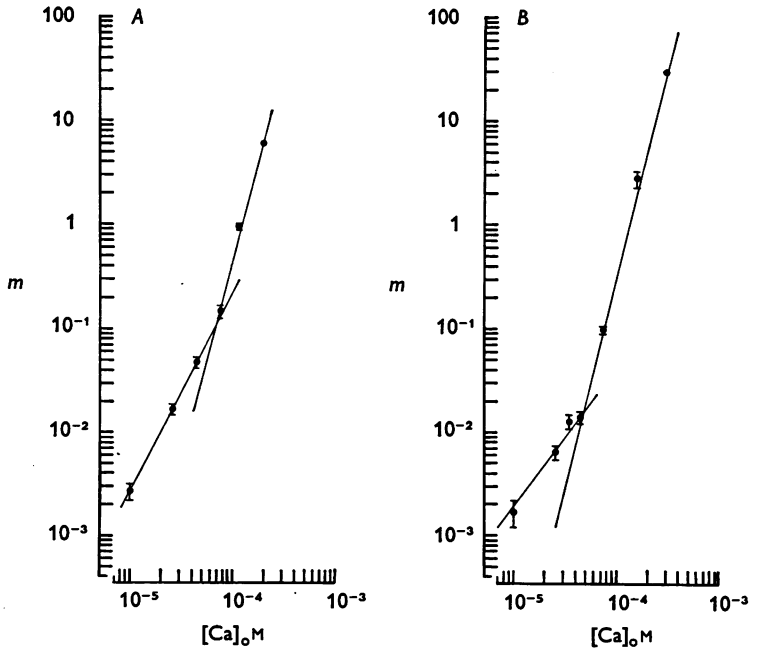

Fig. 5. The calcium dependence of transmitter release in citrate-buffered Ringer. Experiments on two end-plates (A and B) in Ringer buffered with respect to calcium using citrate (mixtures of solutions B and C in Table 1). 5 mM citrate and 5 mM magnesium present throughout. Ordinates: mean quantal content of the end-plate potential ($m \pm$ S.E.). Abscissae: free calcium concentration in the Ringer (M). Note double logarithmic co-ordinates. Temperatures: A 21° C, B 19·5° C.

The simplest explanation of the deviation from a fourth power relationship is that the $[Ca]_o$ in the bath is higher than that nominally contained in the solution. While Murexide assay for free calcium in bath samples

did show a rise in the $[Ca]_0$ from 10^{-5} M to 3×10^{-5} M in Ringer that had been left on the preparation for 6 hr, it was not possible to detect changes of more than 5% over a period of 20 min, the longest time for which any one solution was left on the preparation in these experiments. Although the bulk phase of the solution remains at constant $[Ca]_0$, a calcium leak from the muscle could raise the $[Ca]_0$ in the immediate vicinity of the end-plate. Miledi & Thies (1971) obtained evidence of just such a standing calcium gradient in preparations bathed in a Ringer containing EGTA. Two approaches were used to try to eliminate the effects of local differences in the calcium concentration.

Experiments with citrate-buffered Ringer

A series of experiments was carried out using Ringer containing 5 mM citrate and a total of 5 mM-$MgCl_2$, the $[Ca]_{total}/[citrate]_{total}$ ratio being varied to give the free $[Ca]_0$ required. Solution compositions can be derived from *B* and *C* of Table 1. The buffer curve for citrate under these conditions, as calculated from the stability constants, is given in Fig. 1. It was of interest to buffer calcium in the range 10^{-4}–10^{-5} M. Fig. 1 shows that in this Ringer calcium is buffered quite strongly down to 3×10^{-5} M but below this buffering is probably inadequate.

Typical experiments are shown in Fig. 5*A* and *B*. The buffer concentration was constant and present in all solutions. Points above a $[Ca]_0$ of about 5×10^{-5} M are well fitted by a straight line that approaches a slope of 4 (see Table 2) and the mean quantal content under these conditions behaves as Dodge & Rahamimoff (1967), found for unbuffered solutions. The presence of citrate does not appear to affect transmitter release. Least-square regression fits to the equation

$$m = a_1 [Ca]^{b_1},$$

where a_1 and b_1 are constants, were applied to the upper part of the curve and the values of a_1 and b_1 are given for four experiments in Table 2. The mean value of a_1 was $(2{\cdot}17 \pm 0{\cdot}67$ S.E.$) \times 10^3$ and b_1 averaged $3{\cdot}67 \pm 0{\cdot}15$ (S.E.).

At lower $[Ca]_0$ (below about 6×10^{-5} M) the mean quantal content again deviates from the fourth power line such that more quanta are released. Due to the large sampling error at low values of m this part of the curve is not so well defined as the upper part but the points give a reasonably good fit to another straight line, this time with a slope of about one. The sampling errors shown in Fig. 5 cannot account for the deviation from fourth power kinetics since more than two orders of magnitude more quanta released at $[Ca]_0$ of 10^{-5} M than would be expected from the behaviour of m in the millimolar concentration range.

The approximately linear portion of the curve has been fitted in four experiments to the equation

$$m = a_2[\mathrm{Ca}]^{b_2},$$

where a_2 and b_2 are the proportionality constant and slope respectively of the linear portion of the curve. Values for a_2 (mean $4{\cdot}51 \pm 3{\cdot}89$ S.E.) and b_2 (mean $1{\cdot}28 \pm 0{\cdot}23$ S.E.) are given for four experiments in Table 2.

All experiments show features not seen in unbuffered Ringer (compare Fig. 4 and Fig. 5). The nature of the deviation is always an abrupt change to a straight line of similar slope rather than a smooth curve.

In nominally zero-calcium Ringer m rarely falls below 10^{-2} whereas in citrate-buffered Ringer at the same free calcium concentration (10^{-5} M) m falls well below this (see Fig. 5). These differences support the idea that the smooth deviation from the fourth power line in Fig. 4 may result partially from the local accumulation of calcium around the end-plate. The main result however is that there is a linear component to evoked transmitter release that is only seen at very low mean quantal contents. Since extra quanta are released at low $[\mathrm{Ca}]_0$ it is possible to measure m reasonably accurately at much lower $[\mathrm{Ca}]_0$ than was originally supposed. To try to confirm and extend the citrate results another calcium buffering system was used.

Experiments with EDTA-magnesium buffered Ringer

The calcium buffer EDTA normally buffers in the range $5 \times 10^{-7} - 5 \times 10^{-6}$ M free calcium but magnesium ions are known to shift the curve to high free calcium concentrations (Portzehl *et al.* 1964). Fig. 1 shows the buffer curve for 10 mM-EDTA, 10 mM-Mg^{2+} at pH 7·2, the mixture used in these experiments. Ringer compositions are given in columns *D* and *E* of Table 1. These Ringers are much stronger calcium buffers in the range 5×10^{-6}–5×10^{-5} M than those with citrate; the total buffer concentration is twice that of the citrate experiments, the mid point of the buffer curve lies at 10^{-5} M, and the slope of the line about its mid point is steeper. However, the buffer is not effective above a $[\mathrm{Ca}]_0$ of 7×10^{-5} M and the following experiments omit the EDTA but not the Mg at $[\mathrm{Ca}]_0$ above 10^{-4} M.

Fig. 6 shows two typical experiments using EDTA buffered Ringer. The curves are of the same form as those of the citrate experiments but the whole curve is shifted to the right on the calcium axis since the total magnesium concentration is twice that used in the citrate Ringer (Dodge & Rahamimoff, 1967). The two experiments of Fig. 6 are particularly interesting in that they are junctions with very low m.e.p.p. frequencies where it was possible to follow the mean quantal content down into solutions that contained only 5×10^{-6} M calcium. Another such experiment is

shown in Fig. 7. The linear dependence of m on the $[Ca]_0$ is clearly seen with a sharp transition to a fourth power relationship at higher calcium concentrations.

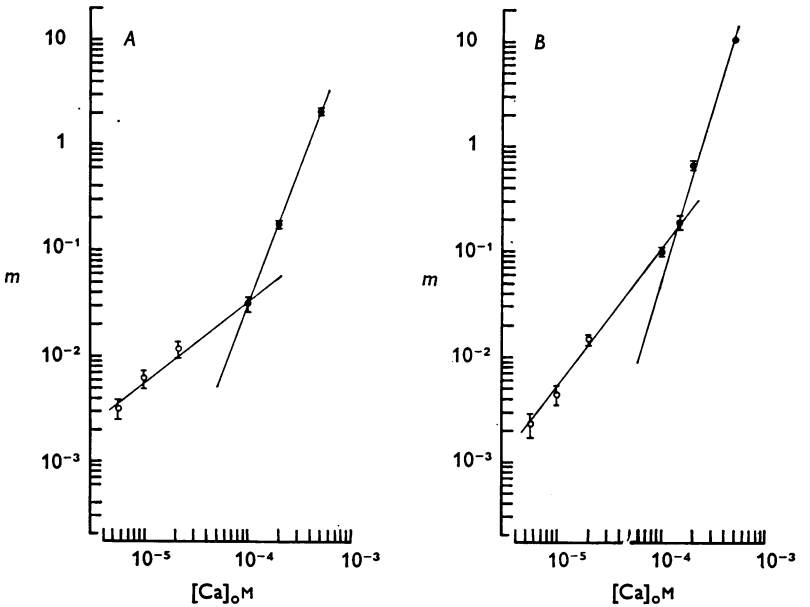

Fig. 6. The calcium dependence of the mean quantal content of the e.p.p. in Ringer buffered for calcium with EDTA. Experiments on two end-plates (*A* and *B*). Open circles indicate solutions buffered with respect to calcium with EDTA and filled circles represent solutions where EDTA was omitted. Buffered solutions contain 10 mM-EDTA and 10 mM magnesium, being formed by mixing solutions *D* and *E* in Table 1. Ordinates: mean quantal content, m ($\pm$ S.E.). Abscissae: free calcium concentration $[Ca]_0$ (M). Note double logarithmic co-ordinates. Temperature: *A* at 20° C, *B* at 21° C.

While attempts were made to underestimate rather than overestimate m (see Discussion) one serious objection has to be raised against these experiments using calcium buffers. Neither buffer is completely selective for calcium over magnesium, the free magnesium concentration falling as the $[Ca]_{total}/[ligand]_{total}$ ratio falls. The buffered solutions contain slightly less free magnesium the lower the free $[Ca]_0$, and this may contribute to the deviation from the fourth power line. No simple way of avoiding this effect could be found but it is possible to minimize it and measure its result. Dodge & Rahamimoff (1967) found that raising the $[Mg]_0$ in the Ringer to a new fixed value caused a shift to the right in the curve relating m to $[Ca]_0$. Magnesium is a competitive inhibitor of the action of calcium in

determining m, with an apparent dissociation constant of about 3 mM. By keeping the $[Mg^{2+}]_o$ in the Ringer high compared to this dissociation constant (K_2) the effects of small changes in the free magnesium concentration should have minimal effect on m. In the EDTA experiments the total $[Mg]_o$ was 10 mM and the free magnesium concentration, even for the lowest $[Ca^{2+}]_o$, never fell below 4·5 mM.

Since it is not known whether the linear part of the curve behaves with

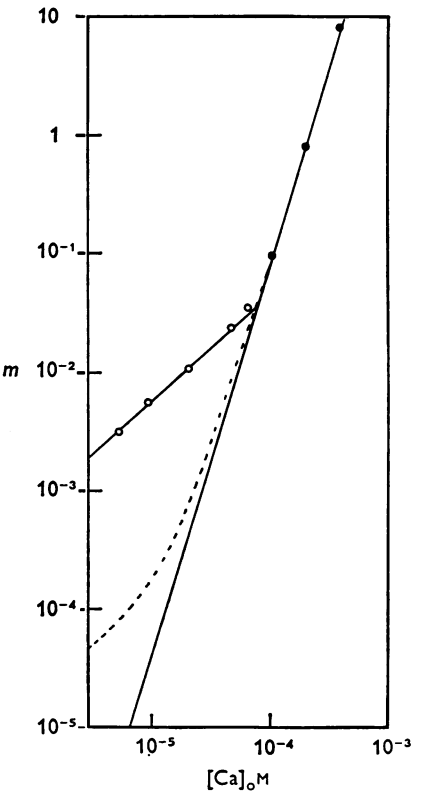

Fig. 7. The effects of changes in the free magnesium in calcium-buffered Ringer on the mean quantal content. Another experiment using EDTA buffered Ringer. Open circles indicate solutions buffered with respect to calcium with EDTA and filled circles represent solutions where EDTA was omitted. Buffered solutions contain 10 mM-EDTA and a total of 10 mM magnesium throughout. Ordinate: mean quantal content ($m \pm$ S.E.). Abscissa: free calcium concentration (M). The line through the upper three points has been extrapolated to lower calcium concentrations. The broken line shows the expected deviation from the continuous line due to change in the free magnesium concentration in the buffer mixtures. It has been calculated from the analysis of Dodge & Rahamimoff (1967). See text for further explanation. For this end-plate K_2 was 3·1 mM. Temperature 22° C.

respect to magnesium as does the fourth power part, it is not valid to correct quantal contents directly for changes in the free magnesium concentration. Fig. 7, another EDTA experiment, shows an attempt to measure the effects of free magnesium changes in the buffer. At the end of the experiment K_2 was determined experimentally for this junction by finding two mixtures of calcium and magnesium that gave the same value of m. K_2 was then calculated (Dodge & Rahamimoff, 1967) from

$$K_2 = \frac{Mg_2Ca_1 - Mg_1Ca_2}{Ca_2 - Ca_1},$$

TABLE 2. Calcium dependence of m in Ringer buffered with respect to calcium with either citrate (C) or EDTA-Mg^{2+} (E). b_1 and b_2 are least-squares regression slopes of the curve in the upper and lower parts respectively. a_1 and a_2 are the proportionality constants of the curve respectively (see text). $(Ca)_t$ is the calcium concentration at transition between the fourth power and linear parts of the curve and m_t the mean quantal content at this point

Expt. no.	Buffer	b_1	a_1	b_2	a_2	Ca_t (mM)	m_t
1	C	4·01	$3·81 \times 10^3$	1·35	0·99	$4·52 \times 10^{-2}$	$1·53 \times 10^{-2}$
2	C	3·32	$1·29 \times 10^3$	1·87	16·16	$4·90 \times 10^{-2}$	$5·79 \times 10^{-2}$
3	C	3·81	$2·70 \times 10^3$	1·12	0·67	$4·54 \times 10^{-2}$	$2·07 \times 10^{-2}$
4	C	3·55	$8·62 \times 10^2$	0·76	0·20	$4·99 \times 10^{-2}$	$2·05 \times 10^{-2}$
5	E	2·52	$1·14 \times 10^1$	0·69	0·76	$2·28 \times 10^{-1}$	$2·74 \times 10^{-1}$
6	E	3·72	$2·64 \times 10^2$	1·32	2·28	$1·38 \times 10^{-1}$	$1·16 \times 10^{-1}$
7	E	3·91	$1·99 \times 10^3$	1·49	14·05	$1·28 \times 10^{-1}$	$6·54 \times 10^{-1}$
8	E	3·05	$1·13 \times 10^2$	0·92	0·40	$7·09 \times 10^{-2}$	$3·52 \times 10^{-2}$
9	E	3·96	$1·93 \times 10^1$	1·93	1·45	$2·79 \times 10^{-1}$	$1·24 \times 10^{-1}$
10	E	4·00	6·30	0·61	0·33	$4·19 \times 10^{-1}$	$1·94 \times 10^{-1}$
Mean ± S.E. (citrate, $n = 4$)		3·67 ± 0·15	$(2·17 \pm 0·67) \times 10^3$	1·28 ± 0·23	4·51 ± 3·89	$(4·74 \pm 0·12) \times 10^{-2}$	$(2·86 \pm 0·98) \times 10^{-2}$
Mean ± S.E. (EDTA, ($n = 6$)		3·53 ± 0·25	$(4·01 \pm 3·21) \times 10^2$	1·16 ± 0·21	0·89 ± 0·33	0·21 ± 0·05	0·23 ± 0·09
Total ($n = 10$)		3·59 ± 0·15	—	1·21 ± 0·15	—	—	—

where Ca_1, Ca_2 and Mg_1, Mg_2 are the concentrations of calcium and magnesium respectively in solutions 1 and 2 that gave the same mean quantal content. For the experiment of Fig. 7 the value of K_2 was 3·2 mM. The continuous line in Fig. 7 is drawn through the fourth power part of the curve and extrapolated to lower calcium concentrations. On the assumption that all of the low-calcium deviation is due to changes in the free $[Mg]_o$ of the buffer, the extrapolated fourth power line has been adjusted for the known changes in free magnesium and the expected deviation is shown

as the broken line. It can be seen that the changes in free magnesium would be expected to exert only a small effect and that only at the lowest calcium concentrations where $[Mg]_0$ begins to approach K_2. Over most of the graph the magnesium effects are smaller than the sampling error in the measurement of m.

Table 2 summarizes the results of ten experiments with the two buffer systems. The column headed $[Ca]_t$ gives the calcium concentration at which transition occurs between the fourth power and linear parts of the curve. This is defined as the point of intersection of the two least-square regression lines for each experiment. The mean value of the high calcium slope (b_1) was not significantly different in citrate Ringer (3·67) from that in the EDTA experiments (3·53) where no buffer was present over this part of the range. These values are slightly lower than those obtained by Dodge & Rahamimoff (1967). The slope of the curve in the approximately linear portion of the curve (b_2) is not significantly different in the citrate experiments (1·28 ± 0·23 S.E.) from the EDTA experiments (1·16 ± 0·21 S.E.) the mean for the ten experiments being 1·21 ± 0·15 S.E. In Table 2 a_1 and a_2 are the extrapolated values for m for the two curves at $[Ca]_0$ in mM equal to 1, and hence determine the position of the curves on the x-axis. The difference in the mean values of a_1 in citrate and EDTA experiments is due to the different magnesium concentrations.

The citrate Ringers contain 5 mM magnesium and the EDTA Ringer 10 mM. magnesium. Dodge & Rahamimoff (1967) found that the interaction of calcium and magnesium could be described by the equation

$$m = K\left(\frac{\frac{X}{K_1}[Ca]}{1+\frac{[Ca]}{K_1}+\frac{[Mg]}{K_2}}\right)^4, \tag{3}$$

where K_1 and K_2 are the apparent dissociation constants of a CaX complex and a MgX complex respectively, K is a proportionality constant relating m to the concentration of CaX, and X is the total concentration of receptor. Thus for two concentrations of magnesium, Mg_1 and Mg_2, giving two values of a_1 (a_1 and a_1' respectively),

$$\left(\frac{a_1}{a_1'}\right)^{1/4} = \frac{K_2+Mg_2}{K_2+Mg_1}. \tag{4}$$

If the change in the value of a_1 from $2{\cdot}17 \times 10^3$ in the citrate experiments to $0{\cdot}4 \times 10^3$ in the EDTA experiments is entirely the result of raising the $[Mg]_0$ from 5 to 10 mM then the mean value of K_2 is 4·4. This figure is similar to the values of Jenkinson (1957), and Dodge & Rahamimoff (1967).

Experiments with cobalt Ringer

While the results from buffered calcium Ringer are in good agreement and independent of the identity or within limits of the concentration of

the buffer, there is no unequivocal way of telling if the calcium concentration in the immediate vicinity of the end-plate is being adequately buffered. Another approach to the original problem was devised by the use of Ringer containing a low concentration of cobalt. The experiments using calcium-buffered Ringer attempt to examine the calcium dependence of very low mean quantal contents. Low values of m are achieved by lowering the external $[Ca^{2+}]$ and hence presumably the calcium entry. Cobalt ions are one of the strongest competitive inhibitors of calcium entry in nerve and at low concentrations have little effect on the action potential (Baker, Hodgkin & Ridgway, 1971; Baker, Meves & Ridgway, 1973). Cobalt is known to reduce reversibly the mean quantal content of the e.p.p. with little effect on the m.e.p.p. frequency at frog end-plates (Kita & Van der Kloot, 1973). The inhibitory action is a competitive one, cobalt being similar to, but about forty times more potent, than magnesium. 4 mM cobalt shifts the calcium dependence curve of m by more than an order of magnitude to the right along the calcium axis, and allows the examination of the calcium dependence of very low mean quantal contents in $[Ca]_0$ that are high enough not to require buffering.

Analysis of the interaction of calcium and cobalt was essentially similar to that of Dodge & Rahamimoff (1967) for the magnesium–calcium interaction (see eqn. (3) above). Assuming that m is directly proportional to the fourth power of the concentration of a complex, CaX, formed between the binding site, X, and calcium, and that cobalt combines also with X to give a complex, CoX, that is ineffective in releasing transmitter, it can be shown that

$$m = K\left(\frac{W.\mathrm{Ca}}{1+\frac{\mathrm{Ca}}{K_1}+\frac{\mathrm{Co}}{K_3}}\right)^4, \tag{6}$$

where K_1 is the dissociation constant of the reaction,

$$\mathrm{Ca}+\mathrm{X} \rightleftharpoons \mathrm{CaX},$$

K_3 is the dissociation constant of the reaction,

$$\mathrm{Co}+\mathrm{X} \rightleftharpoons \mathrm{CoX},$$

Ca and Co are the external concentrations of calcium and cobalt respectively, and W and K are constants.

Fig. 8A shows that the only effect of raising the concentration of cobalt from 0·5 to 4·0 mM is to cause a shift in the calcium dependence curve to the right, i.e. to higher calcium concentrations. Fig. 8B is a Lineweaver–Burke plot of $1/(m)^{\frac{1}{4}}$ against $1/[Ca]_0$ for the same experiment as Fig. 8A. While it is difficult to be sure that the small y-intercept is unchanged in the two cobalt concentrations, the main effect of raising the $[Co]_0$ is obvious; the slope of the graph is increased by cobalt indicating the competitive nature of the calcium–cobalt interaction. The dissociation constant for the CoX complex can be calculated from the concentration of Ca and cobalt in two mixtures that give the same value of m.

$$K_3 = \frac{\mathrm{Co}_2\,\mathrm{Ca}_1-\mathrm{Co}_1\,\mathrm{Ca}_2}{\mathrm{Ca}_2-\mathrm{Ca}_1}, \tag{7}$$

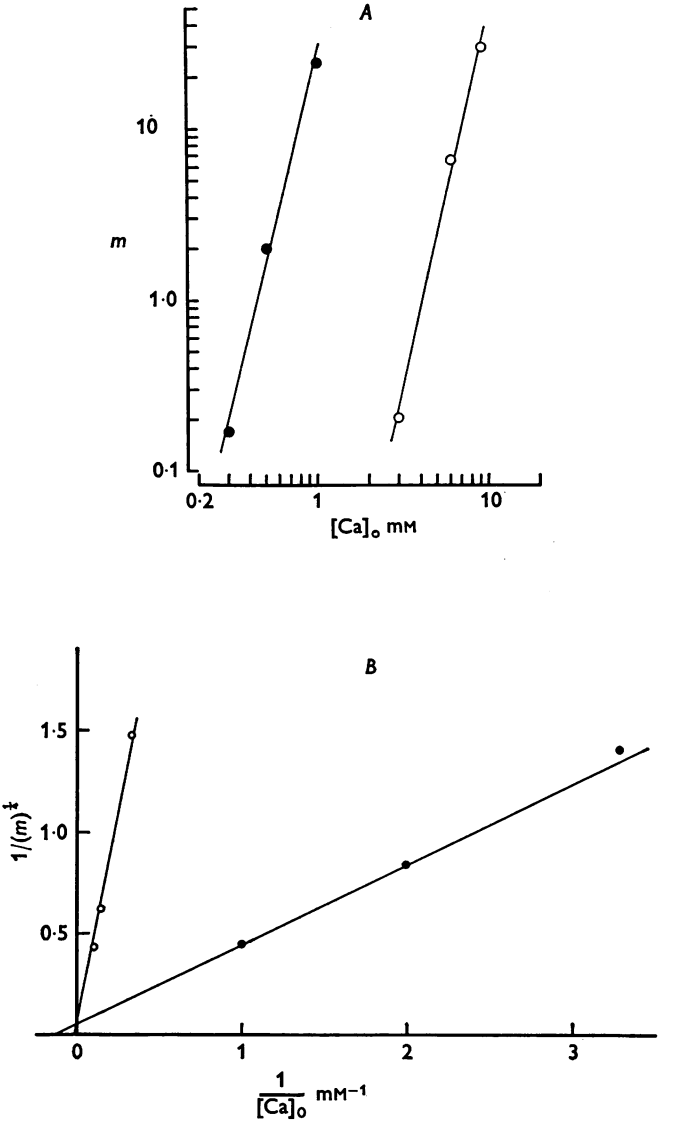

Fig. 8. The competitive interaction of calcium and cobalt in determining m. *A*, double logarithmic plots of m (ordinate) against the external calcium concentration (mM) (abscissa) for two fixed cobalt concentrations. Filled circles 0·5 mM cobalt, open circles 4·0 mM cobalt. All data from the same end-plate. *B*, the same experiment as *A*. Ordinate: $1/(m)^{\frac{1}{2}}$. Abscissa: reciprocal of the external calcium concentration (mM^{-1}). Filled circles 0·5 mM cobalt, open circles 4·0 mM cobalt. Note in this Lineweaver–Burke plot the effect of increasing the cobalt concentration is to increase the slope of the line without changing the intercept on the $1/(m)^{\frac{1}{2}}$ axis. Temperature 19·7° C.

where subscripts 1 and 2 denote ion concentrations in mixtures 1 and 2 respectively. In four experiments the mean value of K_3 was $0{\cdot}07 \pm 0{\cdot}03$ (S.E.) mM. The calculation of K_1, the calcium dissociation constant, involves measuring the intercept Ca* on the negative x-axis of the Lineweaver–Burke plot. Then,

$$K_1 = \frac{\text{Ca}^*}{1 + \frac{\text{Co}}{K_3}}. \tag{8}$$

In four experiments the mean value of K_1 was $1{\cdot}53$ mM $\pm 0{\cdot}45$ (S.E.), a value slightly higher than that found by Dodge & Rahamimoff (1967) which may indicate a slight non-competitive inhibitory action of cobalt such as its known effects in high concentrations on the action potential.

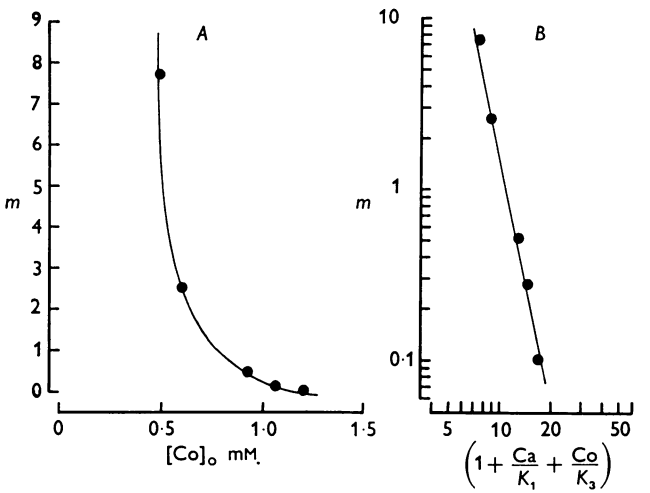

Fig. 9. Cobalt dependence of m at a fixed calcium concentration of 1·0 mM. A: linear co-ordinates; ordinate, mean quantal content; abscissa, external cobalt concentration. B: ordinate, m; abscissa, $(1 + [\text{Ca}]/K_1 + [\text{Co}]/K_3)$ where K_1 is the dissociation constant of the CaX complex and K_3 that of the CoX complex. Note the double logarithmic co-ordinates. For this junction K_1 was 2·01 mM and K_3 was 0·08 mM. Temperature 19·8° C.

Eqn. (6) above predicts that the cobalt dependence of m at a fixed calcium concentration should be a straight line with a slope of -4 on a double logarithmic plot if m is plotted as a function of the denominator. Fig. 9A shows the result of varying the $[\text{Co}]_0$ between 0·5 and 1·2 mM at a fixed calcium concentration of 1·0 mM.

In Fig. 9B the same results are plotted on double logarithmic co-ordinates to illustrate the relationship between m and the denominator of eqn. (6). K_1 and K_3 were determined experimentally for this junction and their values are given in the legend. The expected slope of -4 is seen in Fig. 9B. In two other experiments where the cobalt concentration was raised above 5 mM there was some evidence that the mean quantal content fell off more steeply with cobalt concentration than expected from the hypothesis of simple competition of calcium ions and cobalt ions for the binding site. Since high concentrations of cobalt ions are known to suppress the sodium current of the action potential (Baker *et al.* 1973) a non-competitive

action of cobalt is not surprising. However, below 5 mM the action of cobalt on the mean quantal content seems to be exactly like magnesium but much more potent. K_3 is about one fortieth of the magnesium dissociation constant found by Dodge & Rahamimoff (1967), indicating that cobalt binds to the calcium site forty times as strongly as magnesium. Since it is known that magnesium inhibits transmitter release at the squid giant synapse by reducing the calcium entry during the action potential (Katz & Miledi, 1969) and that cobalt blocks the entry of calcium in squid nerve, it seems likely that the action of cobalt at the neuromuscular junction is similarly mediated by reducing the entry of calcium into the presynaptic terminal.

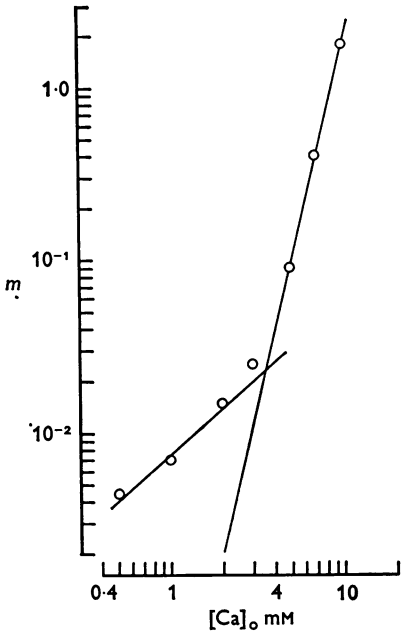

Fig. 10. The calcium dependence of m in Ringer containing a fixed (4·0 mM) concentration of cobalt. Ordinate; mean quantal content. Abscissa: external calcium concentration (mM). The lines through the points are fitted by eye. Note the double logarithmic co-ordinates. Temperature 19° C.

Four experiments were carried out on three preparations in Ringer containing a constant $[Co]_0$ of 4 mM and the calcium concentration was varied between 10 and 0·5 mM by isotonic substitution for sodium (mixtures of solutions *F* and *G* in Table 1). Fig. 10 shows such an experiment. Note that the low values of m (down to about 4×10^{-3}) were achieved in calcium concentrations that do not fall below 0·5 mM. The upper part of the curve shows a slope of 4, but at mean quantal contents below about 5×10^{-2} a deviation occurs until m is an almost linear function of $[Ca]_0$. The form of the curve and the value of m at transition are very similar to the curves in low calcium Ringer. The mean value of the linear part of the curve in four experiments was $1{\cdot}31 \pm 0{\cdot}42$ (S.E.).

DISCUSSION

These experiments show that in low calcium Ringer or in Ringer containing cobalt, when the calcium entry during an action potential is presumably very small, many more quanta are released than are expected from the fourth power behaviour of release at higher calcium concentrations (Dodge & Rahamimoff, 1967; Cooke *et al.* 1973). This extra release is often more than two orders of magnitude more than would be expected.

The measurement of very low mean quantal contents presents several problems, most important of which is the error involved in finite sampling of a Poisson distribution with very low mean. While this error can be reduced with a large enough number of trials, the underlying assumption of stationarity becomes less valid the longer the experiment. In these experiments it was found necessary to come to the compromise of accepting a rather large maximum S.E. of mean (25 %) in order to complete the low calcium measurements in about an hour, a period over which more than 50 % of junctions remained stationary as defined in the Methods.

All junctions bathed in low calcium Ringer for more than an hour showed some reduction of the mean quantal content especially when EDTA-buffered solutions were used. This together with poor sealing of the electrode tip into the muscle fibre in low calcium solutions (Hubbard *et al.* 1968*a*) and the ensuing low m.e.p.p. amplitude probably means that measurements of m are underestimates. Since Miledi & Thies (1971) found that conduction of the action potential into the nerve terminals was largely unaffected at much lower values of calcium ion concentration and at much higher stimulation rates than those used in these experiments, it seems unlikely that conduction block seriously distorted the estimates of m.

The transition from fourth power to linear calcium dependence of the mean quantal content is only clearly seen in low calcium Ringers when the free calcium is adequately buffered. The nature of the transition is again seen with cobalt Ringer under conditions where calcium buffering is not required and at about the same value of m.

The experiments using calcium buffered Ringer show that a rather sudden change from a fourth power dependence of m on $[Ca]_o$ to an approximately linear dependence occurs as the mean quantal content is progressively reduced. EDTA has been widely used to control the calcium concentration inside (Portzehl *et al.* 1964) and outside (Hubbard *et al.* 1968) cells. The main challenge to this and the citrate buffering systems is probably the leakage of calcium out of the muscle fibres (Shanes & Bianchi, 1959). Miledi & Thies (1971) provided indirect evidence that the local concentration of calcium around end-plates can be maintained by

local leakage at more than 2×10^{-8} M even in the presence of 1 mM-EGTA where the buffer should give a $[Ca]_0$ about a fifth of this. In the present experiments the buffer concentrations are much larger than those used by Miledi & Thies (1971), the calcium concentration range is three orders of magnitude higher which would presumably reduce the calcium gradient causing leakage. Unfortunately, since the local conditions at the end-plate are not known, there is no way of stating that the buffers control the local $[Ca]_0$ adequately. However, it seems unlikely that the buffer systems are failing to control the local calcium concentration since the same results are seen in the cobalt experiments. The inclusion of low concentrations of cobaltous ions in the Ringer allows m to be reduced to very low values even in the presence of millimolar concentrations of calcium. Under these conditions it is improbable that the calcium concentration at the end-plate is not that of the bulk solution and yet the calcium dependence curve still linearizes at about the same values of m as occurred with low calcium. The linearization seems to be a genuine property of the release mechanism and not an artifact of inadequate control of the external calcium concentration. Various mechanisms can be proposed for the transition from fourth power dependence of m on $[Ca]_0$ to a linear dependence. If the effective site of action of calcium in release is inside the terminal and this site is accessible to both the resting internal calcium $[Ca]_R$ and the calcium that enters during an action potential, then assuming fourth power kinetics,

$$m = K\{([Ca]_R + \Delta Ca)^4 - ([Ca]_R)^4\}, \quad (1)$$

where K is a proportionality constant relating the instantaneous concentration of internal calcium to m and ΔCa is the change in internal calcium concentration produced by the impulse. It is further assumed that ΔCa is a linear function of the external calcium concentration

$$\Delta Ca = \gamma[Ca]_0, \quad (2)$$

where γ is a constant. This model is similar to that used by Miledi & Thies (1971) to analyse changes in the m.e.p.p. frequency during a tetanus. Its relevant feature is that if ΔCa is large compared to $[Ca]_R$ then

$$m = K\gamma^4 [Ca]_0^4 \quad (3)$$

and as $\Delta Ca/[Ca]_R$ becomes small

$$m \simeq K.4\,[Ca]_R \gamma [Ca]_0. \quad (4)$$

Thus the dependence of m on $[Ca]_0$ changes from a fourth power to a linear relationship at low values of calcium entry. The behaviour results from the location of the site of action of calcium in a compartment available to two sources of calcium, the over-all output of transmitter depending on which source dominates the compartment during an action potential.

There are several objections to this type of explanation. The transition from fourth power to linear calcium dependence produced by the full expansion of equation (1) above is very gradual. Perhaps more convincingly, the model predicts that changes in $[Ca]_R$ at fixed ΔCa should change m only when this is very low. Yet experimental manipulations thought to raise $[Ca]_R$ such as during facilitation (Katz & Miledi, 1968; Weinreich, 1971) and exposure of end-plates to the calcium ionophore nystatin (Crawford & Fettiplace, 1971) produce changes in m at quantal contents high enough to be in the fourth power part of the calcium dependence curve. Even when m has been increased by nystatin treatment the calcium dependence m retains its fourth power dependence on $[Ca]_o$ (Crawford & Fettiplace, 1971).

A variety of other explanations for the fourth power to linear transition can be put forward if one is willing to make assumptions about the way in which calcium binds to its site in the release mechanism. Dodge & Rahamimoff (1967) assumed that all the intermediate complexes of calcium with its binding site other than Ca_4X_4 (or 4CaX) were ineffective in releasing transmitter. One simple way of explaining the appearance of the linear calcium dependence at low values of m would be to accept that intermediate complexes in the formation of Ca_4X_4 did slightly raise the probability of release of a quantum, but very much less than Ca_4X_4. If it is assumed that the dissociation constants (k) of all the four calcium-X reactions are approximately equal and that the proportionality constants relating to the abilities of $[CaX_4]$, $[Ca_2X_4]$ and $[Ca_3X_4]$ to produce release are A and that of $[Ca_4X_4]$ is B, then it can be shown that

$$m = \frac{X_t}{k+[\mathrm{Ca}]}\left\{A[\mathrm{Ca}]\left(1+\frac{[\mathrm{Ca}]}{k}+\frac{[\mathrm{Ca}]^2}{k^2}\right)+\frac{B[\mathrm{Ca}]^4}{k^3}\right\}.$$

where X_t is the total concentration of receptor and if [Ca] is very small this reduces to

$$m = \frac{AX_t}{k}[\mathrm{Ca}]+\frac{BX_t}{k^4}[\mathrm{Ca}]^4.$$

Similar models of release have been proposed by Hubbard *et al.* (1968*a*) and Cooke *et al.* (1973). Almost all the data presented in this paper can be fitted reasonably well to the sum of a linear function and a fourth power function of $[Ca]_o$. However, until more information is available it would be premature to pursue this hypothesis further.

The linear calcium dependence of low quantal contents raises some interesting questions about spontaneous transmitter release. If the m.e.p.p. frequency is determined at least in part by the resting calcium concentration inside the pre-synaptic terminal then the calcium dependence of evoked release when Ca entry is small (and excursions of the $[Ca]_i$ from

the resting calcium concentration are minimal), should describe the calcium dependence of the miniature frequency. It is possible that the m.e.p.p. frequency is a linear function of $[Ca]_i$ rather than the fourth power dependence that Miledi & Thies (1971) assume. Indeed the observation of Hubbard *et al.* (1968*a*) and Cooke *et al.* (1973) that at low $[Ca]_o$ the m.e.p.p. frequency is approximately a linear function of $[Ca]_o$, may be explicable on this basis.

REFERENCES

BAKER, P. F. (1972). Transport and metabolism of calcium ions in nerve. *Prog. Biophys. biophys. Chem.* **24**, 177–223.

BAKER, P. F., HODGKIN, A. L. & RIDGWAY, E. B. (1971). Depolarization and calcium entry in squid giant axons. *J. Physiol.* **218**, 709–755.

BAKER, P. F., MEVES, H. & RIDGWAY, E. B. (1973). Effects of manganese and other agents on calcium uptake that follows depolarization of squid axons. *J. Physiol.* **231**, 511–526.

BROOKS, V. B. (1956). An intracellular study of the action of repetitive nerve volleys and of botulinum toxin on miniature end-plate potentials. *J. Physiol.* **134**, 264–277.

COOKE, J. D., OKAMOTO, K. & QUASTEL, D. M. J. (1973). The role of calcium in depolarization-secretion coupling at the motor nerve terminal. *J. Physiol.* **228**, 459–497.

CRAWFORD, A. C. (1973). Evoked transmitter release at the frog neuromuscular junction in very low calcium solutions. *J. Physiol.* **231**, 47–48*P*.

CRAWFORD, A. C. & FETTIPLACE, R. (1971). A method for altering the intracellular calcium concentration. *J. Physiol.* **217**, 20–21*P*.

CURTIS, B. A. (1963). Some effects of Ca-free choline-Ringer solution on frog skeletal muscle. *J. Physiol.* **166**, 75–86.

DEL CASTILLO, J. & KATZ, B. (1954*a*). Statistical factors involved in neuromuscular facilitation and depression. *J. Physiol.* **124**, 574–585.

DEL CASTILLO, J. & KATZ, B. (1954*b*). Quantal components of the end-plate potential. *J. Physiol.* **124**, 560–573.

DODGE, F. A. & RAHAMIMOFF, R. (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *J. Physiol.* **193**, 419–432.

FRANKENHAEUSER, B. (1957). The effect of calcium on the myelinated nerve fibre. *J. Physiol.* **137**, 245–260.

HODGKIN, A. L. & KEYNES, R. D. (1957). Movement of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.

HUBBARD, J. I. (1963). Repetitive stimulation at the mammalian neuromuscular junction and the mobilization of transmitter. *J. Physiol.* **169**, 641–662.

HUBBARD, J. I., JONES, S. F. & LANDAU, E. M. (1968*a*). On the mechanism by which calcium and magnesium affect the spontaneous release of transmitter from mammalian motor nerve terminals. *J. Physiol.* **194**, 355–380.

HUBBARD, J. J., JONES, S. F. & LANDAU, E. M. (1968*b*). On the mechanism by which calcium and magnesium affect the release of transmitter by nerve impulses. *J. Physiol.* **196**, 75–86.

JENKINSON, D. H. (1957). The nature of the antagonism between calcium and magnesium ions at the neuromuscular junction. *J. Physiol.* **138**, 434–444.

KATZ, B. & MILEDI, R. (1967*a*). On the timing of calcium action during neuromuscular transmission. *J. Physiol.* **189**, 535–544.

KATZ, B. & MILEDI, R. (1967*b*). A study of synaptic transmission in the absence of nerve impulses. *J. Physiol.* **192**, 407–436.

KATZ, B. & MILEDI, R. (1968). The role of calcium in neuromuscular facilitation. *J. Physiol.* **195**, 481–492.

KATZ, B. & MILEDI, R. (1969). Tetrodotoxin resistant electrical activity in presynaptic terminals. *J. Physiol.* **203**, 459–487.

KITA, H. & VAN DER KLOOT, W. (1973). Effects of cobalt and nickel on acetylcholine release at the frog neuromuscular junction. *Nature, New Biol.* **245**, 52–53.

MARTIN, A. R. (1966). Quantal nature of synaptic transmission. *Physiol. Rev.* **46**, 51–66.

MILEDI, R. (1973). Transmitter release induced by injection of calcium into motor nerve terminals. *Proc. R. Soc.* B **183**, 421–425.

MILEDI, R. & THIES, R. (1971). Tetanic and post-tetanic rise in frequency of miniature end-plate potentials in low-calcium solutions. *J. Physiol.* **212**, 245–257.

OHNISHI, T. & EBASHI, O. (1963). Spectrophotometrical measurement of instantaneous calcium binding of the relaxing factor of muscle. *J. Biochem. Tokyo* **54**, 506–511.

PEARSON, E. S. & HARTLEY, H. O. (1966). *Biometrika Tables for Statisticians.* London: Cambridge University Press for the Biometrika Trustees.

PORTZEHL, H., CALDWELL, P. C. & RÜEGG, J. C. (1964). The dependence of contraction and relaxation of muscle fibres from the crab *Maia squinado* on the internal concentration of free calcium ions. *Biochim. biophys. Acta* **79**, 581–591.

RAHAMIMOFF, R. & YAARI, Y. (1973). Delayed release of transmitter at the frog neuromuscular junction. *J. Physiol.* **228**, 241–257.

SHANES, A. M. & BIANCHI, C. P. (1959). The distribution and kinetics of release of radiocalcium in tendon and skeletal muscle. *J. gen. Physiol.* **42**, 1123–1137.

SILLEN, L. G. & MARTELL, A. E. (1964). *Stability Constants of Metal-ion Complexes.* London: The Chemical Society.

WEINREICH, D. (1971). Ionic mechanism of post-tetanic potentiation at the neuromuscular junction of the frog. *J. Physiol.* **212**, 431–446.