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A DUAL EFFECT OF CALCIUM IONS ON NEUROMUSCULAR FACILITATION

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SUMMARY

1. The changes in neuromuscular facilitation produced by varying extracellular calcium and magnesium concentrations have been studied at the frog neuromuscular junction using intracellular recording and automatic averaging of responses.

2. When [Ca] was elevated three effects were observed: a large increase in transmitter release by the first impulse; a decrease in facilitation at short intervals between impulses; and a prolongation of the time course of the facilitated release. If the release by the first impulse is kept at constant level, by raising both [Ca] and [Mg], facilitation becomes greater at all impulse intervals.

3. The results have been discussed in terms of the hypothesis that the action of calcium is responsible for neuromuscular facilitation.

INTRODUCTION

The rate of transmitter release increases very rapidly upon the arrival of an action potential at a motor nerve terminal (Katz & Miledi, 1965*a*). The increased probability of release, which gives rise to the end-plate potential (e.p.p.), does not return immediately to its initial value, but continues for some time afterwards, and is manifested in two ways. First, the frequency of the miniature e.p.p.s is higher after stimulation (Liley, 1956; Brooks, 1956; Hubbard, 1963; Miledi & Thies, 1967) than the spontaneous rate. Further, the amplitude of the second e.p.p. (R_2) evoked shortly after the first (R_1) is greater (Eccles, Katz & Kuffler, 1941; Feng, 1941). This phenomenon of neuromuscular facilitation gradually declines as the interval between the two stimuli is increased, and is often followed

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by a period of depression (Brown & Harvey, 1941; Eccles *et al.* 1941; Lundberg & Quilisch, 1953*a*, *b*; Takeuchi, 1958).

It has been shown by del Castillo & Katz (1954*c*) that facilitation is due to increase in the number of quanta of acetylcholine released from the nerve terminal. This augmented release might partially be due to an increase in amplitude of the second action potential in the presynaptic nerve terminal (Takeuchi & Takeuchi, 1962). However, this does not give a full explanation of the phenomenon, since facilitation has been clearly demonstrated in cases where no increase in the second nerve action potential occurs, and even when the second action potential is reduced in amplitude (Hubbard & Schmidt, 1963; Martin & Pilar, 1964; Katz & Miledi, 1965*b*; Miledi & Slater, 1966).

The striking dependence of the e.p.p. on external calcium concentration led Katz & Miledi (1965*b*) to suggest that facilitation might be due to residual Ca^{2+} left from the first impulse which is still attached to a 'critical site' (X) on the membrane, at the time of the arrival of the second impulse. This possible mechanism of facilitation became even more attractive after the recent finding that a co-operative action of about four calcium ions is necessary in order to produce unit release (Dodge & Rahamimoff, 1967*a*, *b*)

According to this co-operative hypothesis, only 'sites' combined with four Ca^{2+} ions are effective in release. 'Sites' containing three, two or one Ca^{2+} ions are ineffective. The additional assumption necessary in order to apply this hypothesis to facilitation would be that the inactivation of *all* CaX takes many milliseconds. On the arrival of the second action potential, it would be then easier for sites with partial occupation by Ca to reach the required four Ca^{2+} ion level and so induce release of transmitter.

In view of this hypothesis it was of interest to examine the effect of [Ca] on neuromuscular facilitation, in particular under conditions of low quantal content, where no complications arise due to neuromuscular depression (Lundberg & Quilisch, 1953*a*, *b*).

METHODS

The experiments were performed on the sartorius nerve-muscle preparation of the English frog (*Rana temporaria*) at room temperature (19–23° C). Bathing fluid compositions, the method of changing solutions, electrical recording and stimulation were similar to those described previously (Dodge & Rahamimoff, 1967*b*).

Most of the experiments were made under conditions of low quantal content and therefore fluctuating response (del Castillo & Katz, 1954*b*). In order to reduce the sampling error, the automatically averaged response to 64–1024 double stimuli was used to estimate neuromuscular facilitation F ($F = \overline{R}_2/\overline{R}_1$, or $F = m_2/m_1$), where $\overline{R}_1$ and $\overline{R}_2$, m_1 and m_2 are the

average e.p.p. or quantal content of the first and the second response respectively. In each experiment the number of double stimuli (n) was chosen so as to reduce the standard error of facilitation (P), well below 5 % of the mean, using the following equation:

$$n = 1{\cdot}1\left(\frac{1}{\bar{R}_1}+\frac{1}{\bar{R}_2}\right)\frac{\bar{a}}{\bar{P}^2}, \tag{1}$$

where $\bar{a}$ is the average amplitude of the miniature e.p.p. (m.e.p.p.) (see Rahamimoff, 1967). Such a procedure of working to a predetermined level of sampling error was found necessary, since the effects to be described are relatively small.

There was a considerably variability in the degree of facilitation. This was observed, not only among different preparations, where some uncontrolled factor might be involved (e.g. resting tension—see Hutter & Trautwein, 1956), but also from end-plate to end-plate in the same muscle (Fig. 1). Therefore, each experiment was carried out on the same end-plate, with various Ringer compositions. E.p.p. amplitudes were corrected for non-linear summation (Martin, 1955).

RESULTS

If neuromuscular facilitation (F) is due to residual Ca left on critical sites of the nerve terminal by the first impulse, it is possible to predict that F should decrease with increase in [Ca], for increase in [Ca] causes more critical sites to be occupied by the first impulse (hence an increase in m) and the fraction of residual sites available for the second impulse would be relatively smaller (see Discussion, eqn. 7). It should be noted that this prediction applies to the case of a steady concentration of external Ca, which remains the same during the arrival of the first and the second impulses. An entirely different situation arises if, by iontophoretic pulse applications, [Ca] is altered during the arrival of the two impulses (see Katz & Miledi, 1968).

The experimental results reveal that the above prediction is only partially fulfilled (Figs. 2, 3). Raising [Ca] causes a decrease in facilitation (Figs. 3A, 4) as expected from the above prediction, when the time intervals between the two stimuli ($\bar{N}_1\bar{N}_2$) are short, but at longer $\bar{N}_1\bar{N}_2$ the opposite effect is found (Fig. 3*B*).

These results show that Ca^{2+} ions affect not only the magnitude of facilitation, but also its time course. At higher [Ca] the rate of decay of F is slower, and the facilitation *vs.* $\bar{N}_1\bar{N}_2$ curves cross each other. Similar results to those in Fig. 2 were obtained from four additional end-plates. The point of intersection varied in different experiments between 10 and 40 msec. It is of interest to note that such an intersection can be seen also in earlier work (Lundberg & Quilisch, 1953*b*, their fig. 5).

It is possible that part of the dual effect arises from the very marked increase in release associated with raising [Ca]. The procedure was therefore modified so as to keep the release by the first impulse at an approximately constant level. This was done in the following way: first a facilita-

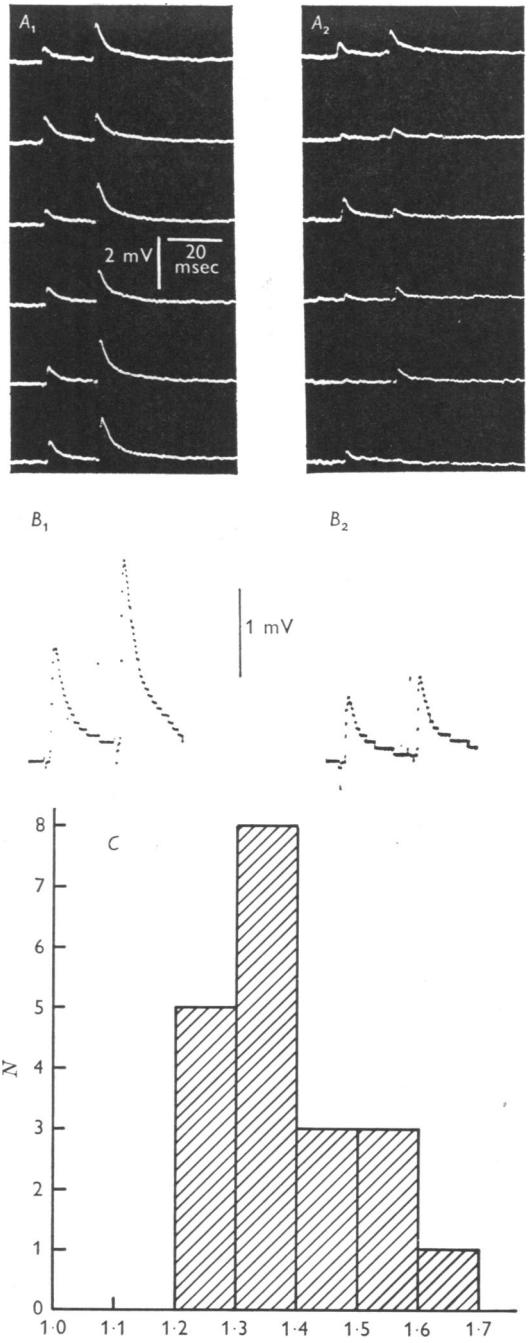

Fig. 1. For legend see opposite page.

tion curve was obtained in a Ringer solution containing low Ca^{2+} and Mg^{2+} ($[Ca]_1$, $[Mg]_1$). Then, [Ca] was raised to $[Ca]_2$ and [Mg] was adjusted so that the first stimulus produced approximately the same release as before. An estimate of the required $[Mg]_2$ was obtained by using the following equations:

$$m_1 = K\left(\frac{W[Ca]_1}{1+\frac{[Ca]_1}{K_1}+\frac{[Mg]_1}{K_2}}\right)^4 = K\left(\frac{W[Ca]_2}{1+\frac{[Ca]_2}{K_1}+\frac{[Mg]_2}{K_2}}\right)^4, \qquad (2)$$

where W is a constant, K a proportionately constant, and K_1 and K_2 dissociation constants of the equations $Ca+X \rightleftharpoons CaX$ and $Mg+X \rightleftharpoons MgX$, respectively.

Therefore

$$[Mg]_2 = K_2\left[\frac{[Ca]_2}{[Ca]_1}\left(1+\frac{[Mg]_1}{K_2}\right)-1\right]. \qquad (3)$$

The only unknown factor on the right-hand side of eqn. (3) is the dissociation constant for Mg, K_2. Its value was not determined in the present experiments, and the average value of 3·0 mM (S.D. ± 0·76) reported previously (Dodge & Rahamimoff, 1967*b*) was used as a trial. It was frequently necessary to readjust $[Mg]_2$ in order to obtain the same m_1.

Figure 5 illustrates the results of such an experiment. The lower curve was obtained in Ringer containing 0·5 mM-Ca^{2+} and 1·0 mM-Mg^{2+}, with average quantal content for the first response of 6·1. The upper curve was obtained in 1·5 mM-Ca^{2+} and 8·0 mM-Mg^{2+}. $\bar{m}_1$ was 6·4. The two curves differ significantly from each other, indicating that the increase in divalent ion concentration causes both the amplitude and the duration of the facilitation process to increase, provided that the initial release remains constant. Similar results were obtained from three additional junctions.

With this experimental procedure Ca and Mg were substituted for Na in order to keep the osmolarity constant. This resulted in a reduction of [Na] of up to 15 %. As a control, one-fifth of the [Na] was replaced by isos-

Legend for Fig. 1.

Fig. 1. Neuromuscular facilitation at different end-plates in the same nerve muscle preparation. A_1 and A_2 are sample records from two different end-plates. B_1 and B_2 are the corresponding averaged records. B_1 is an average of 128 double responses, and B_2 of 512 double responses. Averaging step 160 μsec. *C* is from the same muscle, a histogram showing the distribution of facilitation among twenty different superficial end-plates. Each estimation of facilitation based upon 64-1024 double responses. The mean facilitation is 1·396 (S.D. + 0·15). There is no dependence on the sequential number (X) of the examined end-plate, the regression equation being $F = -0{\cdot}0015X + 1{\cdot}41$. The medium contained 0·3 mM-Ca^{2+} and 1·5 mM-Mg^{2+} throughout. Time interval 20 msec. Temperature approx. 20° C.

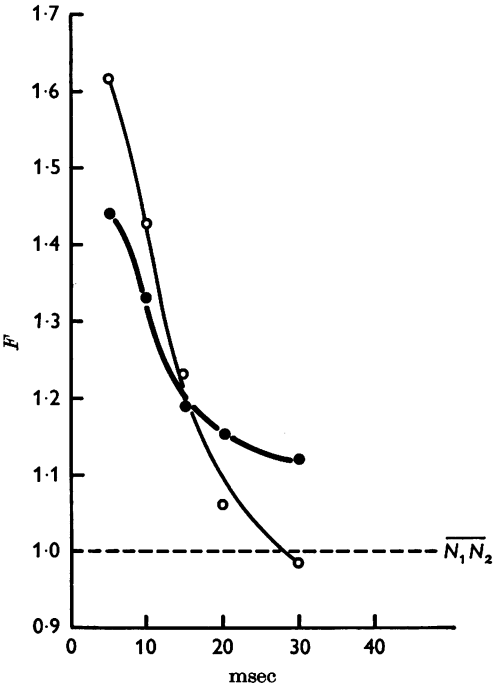

Fig. 2. Time course of facilitation. Ordinate: F, the ratio of $\overline{R}_2$ over $\overline{R}_1$. Abscissa: Interval between N_1 and N_2. The two curves were obtained at the same end-plate at two different [Ca]. ○, 0·2 mM-Ca^{2+}; ●, 0·4 mM-Ca^{2+}. 1·0 mM-Mg^{2+} present throughout the experiment. Note the intersection of the curves. Interrupted line indicates equal release by the first and the second impulse. F = 1·0.

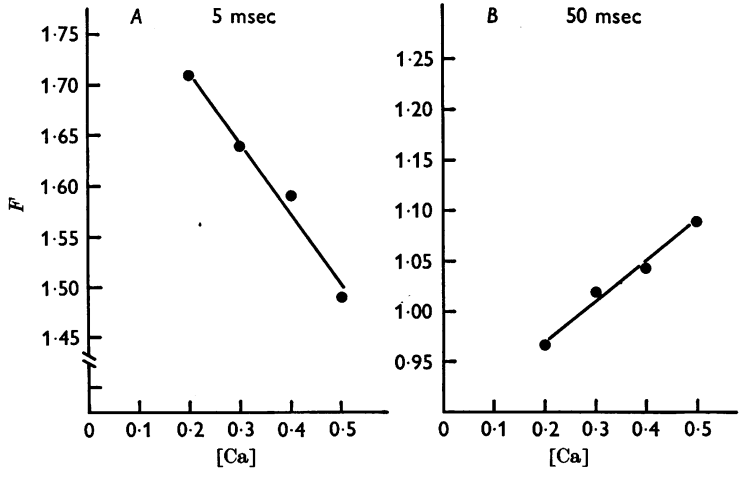

Fig. 3. Effect of calcium on facilitation at two different $\overline{W}_1\overline{W}_2$. *A*, 5 msec; *B*, 50 msec. *A* and *B* obtained from the same end-plate. 1·0 mM-Mg^{2+} present throughout the experiment.

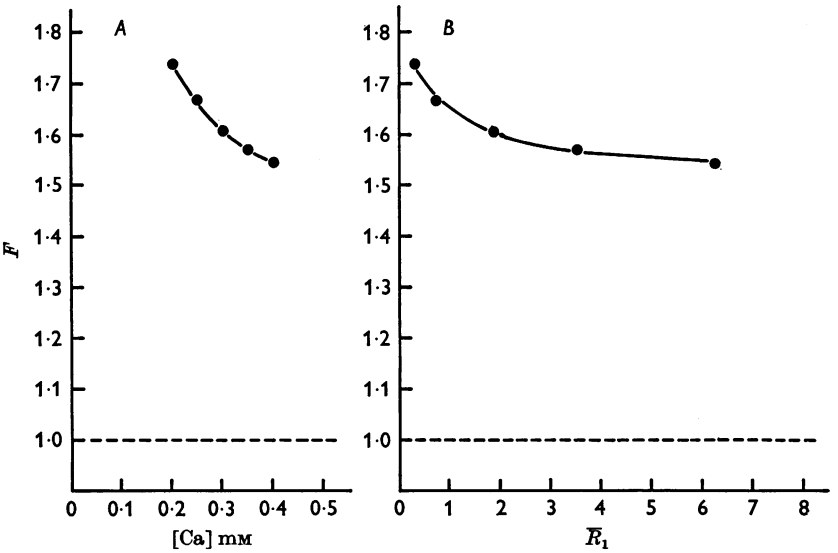

Fig. 4. Changes in facilitated release with [Ca] (*A*) and with the amplitude of the first e.p.p. (*B*). All points from the same end-plate. The number of double responses averaged for each point are (from left to right) 512, 512, 256, 256, 128. Time interval between the two stimuli 7 msec. 1·0 mM-Mg^{2+} present throughout.

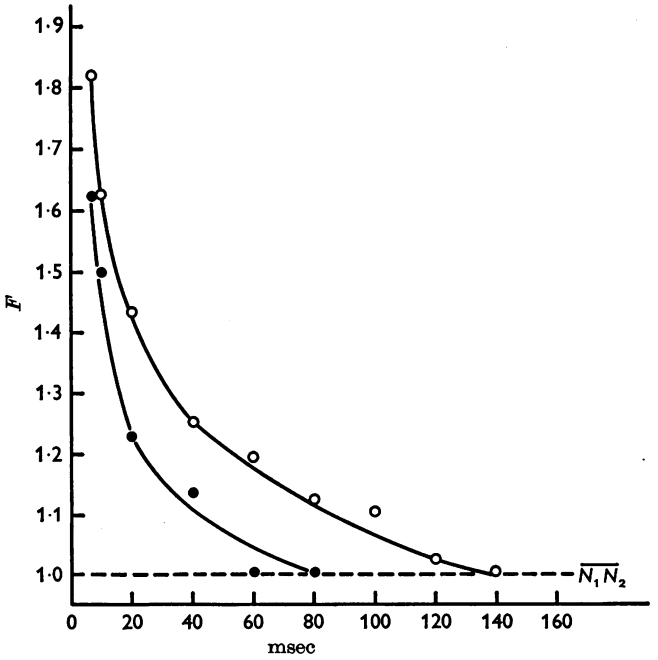

Fig. 5. Facilitation *vs.* N_1N_2 at two different divalent ion compositions of the medium that produce equal release by the first stimulus. ●, 0·5 mM-Ca^{2+} and 1·0 mM-Mg^{2+}; ○, 1·5 mM-Ca^{2+} and 8·0 mM-Mg^{2+}. Each point is an average of 256 double responses (see text).

motic sucrose without changing [Ca] and [Mg]. This produced no apparent effect on F.

It appears, therefore, that Ca ions have at least two different effects on neuromuscular facilitation. When the release due to the first stimulus is kept constant, increase in [Ca] increases the duration of facilitation. When the release is allowed to increase, the facilitation at short intervals becomes depressed. This dependence of F on the released amount can also be demonstrated in a somewhat different way: release could be suppressed by raising [Mg] without altering [Ca]. At six end-plates facilitation was examined with two pulses separated by 10 msec, in Ringer solutions containing 0·4 mM-Ca^{2+} and either 1·0 mM-Mg^{2+} or 8·0 mM-Mg^{2+}. At 8·0 mM-Mg^{2+} facilitation was increased on the average by 29 % (range 12–41 %).

The decline in F with increased [Ca] is observed when facilitation is measured as the *ratio* between the second and the first e.p.p. If one considers the *absolute* increase in release (i.e. the *difference* between second and first e.p.p.), this grows with increase in [Ca].

DISCUSSION

The present experiments are concerned only with an early component of neuromuscular facilitation. A later component which was recently demonstrated by Mallart & Martin (1967) was not studied here.

Two main actions of Ca ions were found: increase of [Ca] causes the decay of facilitation to become slower, and the size of initial facilitation decreases with increase in m_1. This second observation is to be expected from the calcium hypothesis for neuromuscular facilitation (Katz & Miledi, 1965*b*). To illustrate this point, let us assume that there are critical sites 'X' on the nerve terminal that combine with Ca giving CaX (see del Castillo & Katz, 1954*a*; Jenkinson, 1957; Dodge & Rahamimoff, 1967*b*). During the first impulse the fraction of sites $-A$, occupied by Ca would be given by

$$A = \frac{w\mathrm{Ca}}{1+\frac{\mathrm{Ca}}{K_1}+\frac{\mathrm{Mg}}{K_2}}, \tag{4}$$

and the fraction not occupied would $1-A$. If it is further assumed that CaX decays with time constant α, then the first and the second responses would be given by

$$m_1 = KA^4, \tag{5}$$

$$m_2 = KA^4(1-A\,e^{-\alpha t}+e^{-\alpha t})^4, \tag{6}$$

and facilitation $F = m_2/m_1$

$$F = [1+e^{-\alpha t}(1-A)]^4 \tag{7}$$

It follows from eqn. 7 that increase in A (associated with increase in m) would tend to decrease facilitation. This is what happened when A (or m) is changed by lowering Mg, or when the change in initial facilitation with rising [Ca] is considered. Similar results concerning the dependence of F on m have been obtained by A. R. Martin during a study of Mg action (personal communication). It is of interest to note that Thies (1965) obtained a decrease in facilitation with increase in m, in the serratus nerve muscle preparation of the guinea-pig. However, in his experiments m was much larger than in the present and Martin's work. This raises the possibility of a further complicating factor, namely that in Thies's experiments the observed decrease in facilitation at higher values of m was due to the intervention of neuromuscular depression which becomes marked at high release rates. Such an explanation does not apply to the present experiments, since only very few quanta were released per impulse (see also Lundberg & Quilisch, 1953*a*, *b*).

It should be pointed out that eqn. 7 does not represent fully the events which follow the arrival of an impulse. For example, according to eqn. 7, the maximal degree of facilitation is 16 (when $t \to 0$ and A is small, then $F \to 16$). However, Katz & Miledi (1968) have found in a tetrodotoxin treated preparation that the second of two depolarizing pulses if delivered to the terminal after a very brief interval (0–1·0 msec), often caused a more than 16-fold increase in release than the first pulse alone. An additional factor that complicates the direct application of eqn. 7 is that α is dependent on the extracellular divalent ion concentration.

There are some indications that the decay of the high probability state that follows the e.p.p. might involve an active, metabolism-dependent process rather than a passive decay. Thus, the decay of facilitation has a high temperature coefficient. Lowering the temperature prolongs the facilitation, the Q_{10} being more than 3 (Eccles *et al.* 1941). Furthermore, it has recently been found that ouabain increases facilitation (F. Colomo & R. Rahamimoff, unpublished data).

In conclusion, the present experiments show that neuromuscular facilitation follows some of the predictions arising from the hypothesis that residual calcium ions combined with membrane sites are responsible for the augmented release by a second impulse. However, for a more complete understanding of this phenomenon, an elucidation of additional factors will be necessary.

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