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THE TIMING OF CALCIUM ACTION DURING NEUROMUSCULAR TRANSMISSION

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SUMMARY

1. When a nerve–muscle preparation is paralysed by tetrodotoxin, brief depolarizing pulses applied to a motor nerve ending cause packets of acetylcholine to be released and evoke end-plate potentials (e.p.p.s), provided calcium ions are present in the extracellular fluid.

2. By ionophoretic discharge from a 1 M-$CaCl_2$ pipette, it is possible to produce a sudden increase in the local calcium concentration at the myoneural junction, at varying times before or after the depolarizing pulse.

3. A brief application of calcium facilitates transmitter release if it occurs immediately before the depolarizing pulse. If the calcium pulse is applied a little later, during the period of the synaptic delay, it is ineffective.

4. It is concluded that the utilization of external calcium ions at the neuromuscular junction is restricted to a brief period which barely outlasts the depolarization of the nerve ending, and which precedes the transmitter release itself.

5. The suppressing effect of magnesium on transmitter release was studied by a similar method, with ionophoretic discharges from a 1 M-$MgCl_2$-filled pipette. The results, though not quite as clear as with calcium, indicate that Mg pulses also are only effective if they precede the depolarizing pulses.

INTRODUCTION

The presence of extracellular calcium ions is known to be essential for neuromuscular transmission. The principal point of calcium action is the process by which the nerve impulse releases acetylcholine from the motor nerve endings (Katz & Miledi, 1965*c*). This process can be studied even when the nerve impulse and its accompanying sodium current have been eliminated by tetrodotoxin; under these conditions a brief depolarizing pulse locally applied to the nerve ending causes 'packets' of acetylcholine to be released, provided calcium ions are present in the extracellular medium (Katz & Miledi, 1967*a*). The time course of the transmitter release could be determined with great accuracy, by measuring the statistically

varying intervals between pulse and quantal e.p.p.s in a long series of observations. After a brief pulse (0·5–1 msec), there is a short delay, about 1–2 msec at 5° C, during which the probability of release does not perceptibly exceed the low background level. This is followed by a rapid rise to a peak and a gradual decline of the probability of release which may extend over 10 msec or more.

It was of interest to find out at what stage during this sequence of events the external calcium ions come into play. By close ionophoretic application, it is possible to produce a sudden increase in the local calcium concentration, and to time it fairly accurately in relation to the depolarizing pulse. The question which the present experiments are meant to answer is whether the extracellular calcium is utilized during the period preceding the transmitter release (i.e. during depolarization plus initial latency), or whether it becomes effective during the transmitter release itself.

METHODS

The procedure follows that described in previous papers (Katz & Miledi, 1965*a*, *d*; 1967*a*, *b*). The muscle (sartorius of *Rana pipiens* or *temporaria*) was placed in a Ringer solution of low calcium (< 0·1 mM) and added magnesium (about 1 mM) content, paralysed by tetrodotoxin (about 10^{-6} g/ml.) and kept at low temperature (about 4° C). The main difference from previous work is that a twin-pipette was employed, containing 1 M solutions of NaCl and $CaCl_2$ respectively in the two barrels. The sodium channel was used to apply depolarizing and various electrical control pulses to the surface of the nerve terminal, while the other channel was used to raise the local calcium concentration at desired moments. Once a calcium effect was seen, the strength and duration of the pulse was reduced and the pipette carefully re-positioned until an optimum effect was obtained. E.p.p.s were recorded with an intracellular electrode. Prostigmine (10^{-6} g/ml.) was used in most experiments. In other experiments, twin- or triple-pipettes were used one of whose barrels contained 1 M-$MgCl_2$ replacing, or in addition to, the calcium pipette.

The use of multiple-barrel pipettes made special precautions necessary. A strong current pulse through one of the barrels was apt to cause a transient resistance change in an adjacent barrel, possibly by dislodging charged particles from the tip. This was checked by monitoring the current intensities, and it was verified that, usually, barrel interactions of this kind were not large enough to vitiate the results.

When applying a strong positive-going pulse to the calcium pipette, the possibility of electrical (as distinct from ionophoretic) effects had to be considered (see Katz & Miledi, 1967*b*, Methods). Certain control experiments have already been reported (Katz & Miledi, 1967*b*): depolarizing pulses were followed after intervals of 20–140 μsec by similar hyperpolarizing (positive-going) pulses from the same sodium pipette. It was shown that there is no significant interference by the positive pulse, if it was applied more than 100 μsec after the end of the depolarization, and only a small reduction of the transmitter release when applied within 20–50 μsec. These earlier findings are also relevant to the observations described below.

RESULTS

Figures 1 and 2 illustrate results of two experiments. A series of depolarizing pulses (*P*) was applied via the sodium barrel, at intervals of several seconds. The pulses given by themselves failed to evoke more than

very infrequent unit e.p.p.s. If P was preceded by a brief ionophoretic discharge of calcium from the other barrel (positive-going pulse Ca), the failure rate was greatly reduced, and unit responses occurred at varying times as shown in Figs. 1–4. (Italicized symbols (Ca, Mg) refer to ionophoretically applied doses of these substances.)

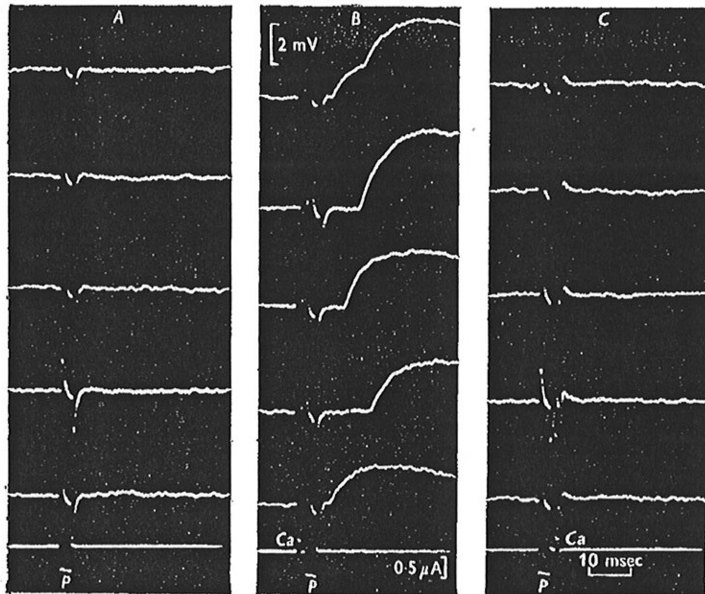

Fig. 1. Effect of ionophoretic pulses of calcium (Ca) on end-plate response. Depolarizing pulses (P), and calcium were applied from a twin-barrel micropipette to a small part of the nerve–muscle junction. Intracellular recording from the end-plate region of a muscle fibre. Bottom traces show current pulses through the pipette. Column A. Depolarizing pulse alone. B. Calcium pulse precedes depolarizing pulse. C. Depolarization precedes calcium pulse. Temperature 4° C.

To obtain a maximum effect, the interval between Ca and P pulses had to be adjusted so as to allow the calcium concentration to reach a peak at the critical site and time. In the case of Fig. 5, the optimum Ca–P interval was about 10–20 msec. But a significant effect could be obtained with much shorter intervals, and this was important for the present study. With careful placing of the pipette, a facilitating action of calcium could be observed when a Ca pulse as brief as 1 msec was applied, separated from the start of the depolarizing pulse by as little as 50–100 μsec.

In the experiments illustrated in Figs. 1 and 2, pairs of $Ca+P$ pulses alternated with pairs in which the time sequence of Ca and P was reversed. The result was unequivocal: Ca pulses given *after* the depolarization were ineffective. They failed to raise the level of response even if they were

applied immediately after P, i.e. during the minimum latent period of the recorded response. It should be noted that it was not possible to synchronize the Ca and P pulses, because simultaneous application of the two pulses of opposite sign cancelled some of the depolarization produced by P alone.

One may conclude from these experiments that the utilization of external calcium ions occurs during, and possibly immediately after, the depolarization, that is during a period preceding that of increased probability of transmitter release. It is true that the time resolution of the ionophoretic method is limited because of inevitable diffusion delay. Nevertheless, the difference in timing between effective Ca pulses, immediately before P,

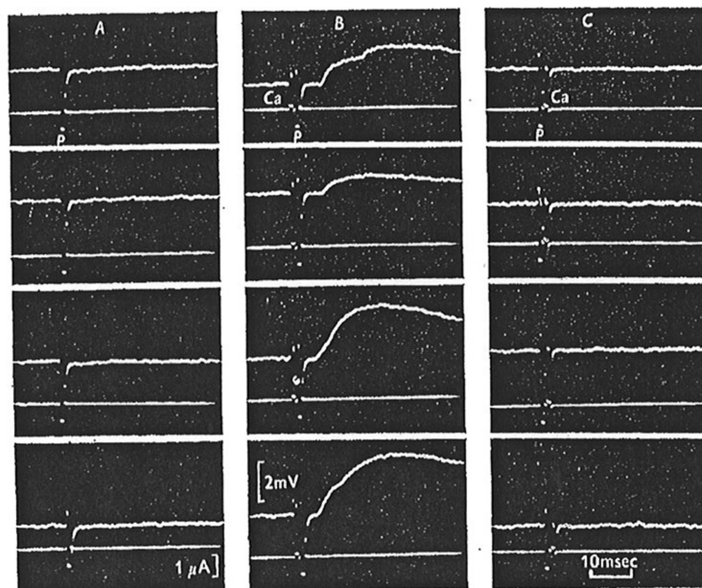

Fig. 2. A, depolarizing pulses (P); B, Ca pulses $+P$; C, $P+Ca$. The currents through the twin-pipette are shown in the bottom trace of each record. Temperature 3° C.

and ineffective Ca pulses, after P, was so small that our interpretation cannot be in doubt. If one were to argue that the responses obtained with $Ca+P$ are due to external calcium ions reaching the critical membrane sites only after the initial latent period, then there would be no explanation for the fact that calcium ions discharged ionophoretically during the latency fail to promote the occurrence of later units of response (Figs. 3, 4).

A number of controls were made to check on any electric interaction between the two barrels. In addition to the observations mentioned in Methods, a test was made to see whether the negative P pulse from the sodium barrel interfered in any way with the discharge of calcium by an

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immediately following pulse from the other barrel. This point was examined by giving a triple sequence of pulses $P_1 + Ca + P_2$, and comparing the effect with the usual $Ca + P$ action (relating, in each case, the response to that obtained without the Ca pulse). The result is shown in Table 1. The calculated values of 'm' are not at all accurate, but there was clearly no evidence for any substantial reduction of the calcium effect by the immediately preceding negative pulse P.

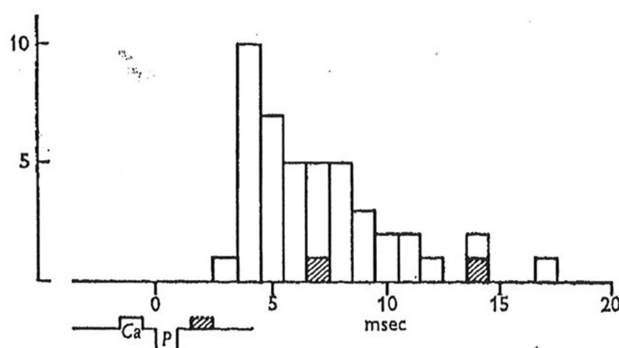

Fig. 3. Histogram of 'synaptic delays'. Abscissa: time interval between start of depolarizing pulse (P) and beginning of a unit end-plate potential. The time relations between calcium and depolarizing pulse are indicated below. Ordinate: number of observed unit potentials. The main histogram shows responses evoked by forty-nine ($Ca + P$) pulses. Shaded blocks: responses evoked by 48 ($P + Ca$) pulses. (These were as infrequent as responses due to P alone.) Temperature 3° C.

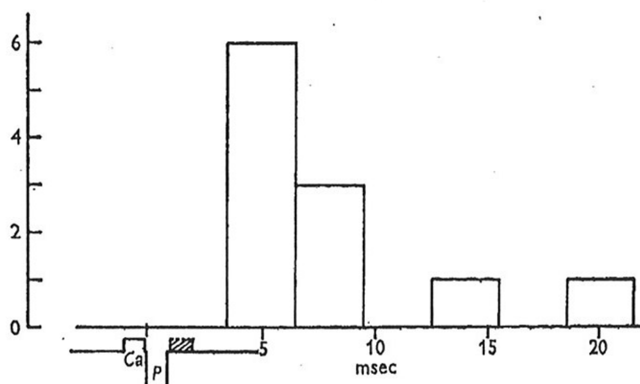

Fig. 4. Another histogram, obtained with very brief separation between P and Ca pulses. $Ca + P$ gave eleven unit responses (as shown) to twenty-nine pulses. $P + Ca$ (see shaded Ca block below) produced no response in fourteen trials. P alone also failed to evoke any response. Temperature 4·5° C.

Another test was to verify that a *Ca* pulse following *P* did not *reduce* the response. This was done simply by raising the steady Ca-efflux from the pipette (see Katz & Miledi, 1965*a*, *c*) and repeating the experiment with an increased initial rate of *P* responses.

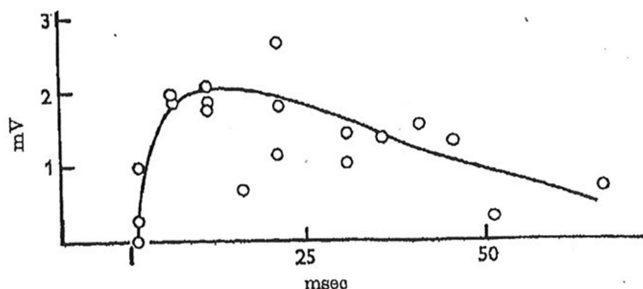

Fig. 5. Effect of time interval between *Ca* and *P* on end-plate response. Abscissa: Interval between start of *Ca* pulse (1 msec duration) and 2·7 msec depolarizing pulse *P*. Ordinates are amplitudes of individual e.p.p.s evoked by *P*. Temperature 3° C.

TABLE 1. Control experiment

A B *Ca* E F; C D; P_1 P_2

AB, *EF* 1·9 msec; *AC* 2 msec; *CD*, 1·7 msec; *AE* 7 msec.

	P_2	$Ca+P_2$	P_1+P_2	P_1+Ca+P_2
(Responses)/(pulses)	1/13	5/12	8/26	9/10
'*m*' [= ln (pulses)/(failures)]	0·08	0·54	0·37	2·3

In a few experiments, the inhibitory influence of magnesium on transmitter release was studied by ionophoretic application (Figs. 6–8). Magnesium is less potent in equimolar concentration than its antagonist calcium, and as a consequence it was difficult to produce a sufficiently intense suppression by brief ionophoretic discharges from the *Mg*-barrel. Indeed, the positive pulses had to be made so strong that local membrane break-down was a serious risk (see Katz & Miledi, 1967*b*). It was possible, nevertheless, to obtain clear inhibitory effects with *Mg* pulses of 5 msec duration provided they were applied *before* *P*. Figs. 7 and 8 illustrate an experiment with a triple pipette (the three barrels containing respectively, Ca, Na and Mg). A *Ca* pulse was given throughout, preceding *P* by about 15 msec, in order to elicit a background rate of response sufficiently high for testing the suppressing action of *Mg*. Figure 8 combines the results of two complete series in which somewhat different interval settings for *P* and *Mg* pulses were chosen. The bottom part shows the responses to eighty-three *P* pulses in the absence of magnesium. When *Mg* pulses preceded *P*,

the average value of m (i.e. ln (number of pulses)/(number of failures)) was reduced from 0·96 to 0·22, and from 0·4 to 0·03 in the two series, respectively. The residual responses (to sixty-one pulses) are represented in the histogram at the top (Fig. 8A). When the Mg pulses *followed* P (middle histogram, Fig. 8B, sixty-five pulses), no suppression was observed.

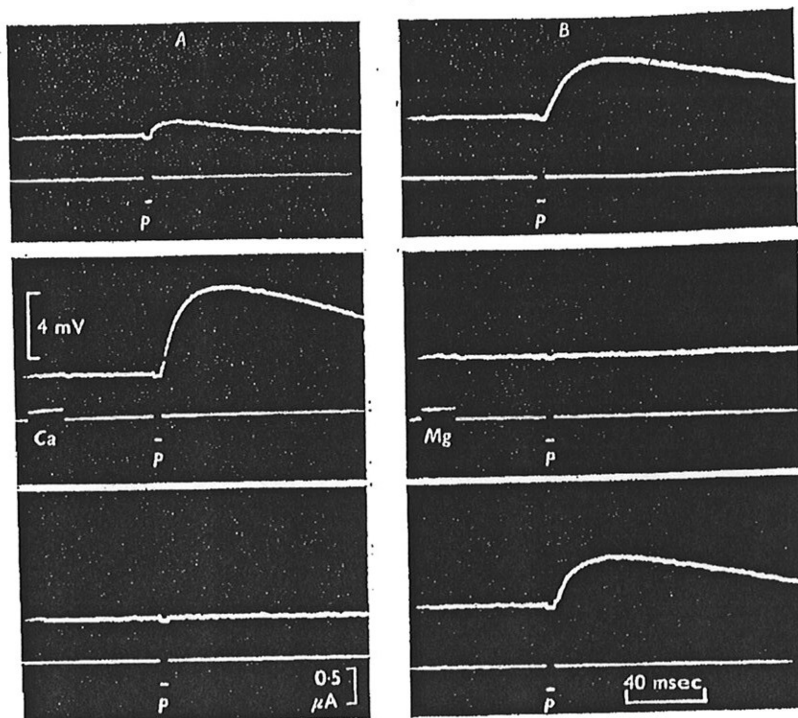

Fig. 6. A triple-barrel pipette containing, respectively, Ca, Na (for depolarization P), and Mg, was used. Column A shows the facilitating action of a calcium pulse; column B shows the inhibiting influence of a magnesium pulse. To demonstrate the inhibitory effect, the bias on the calcium barrel was adjusted between A and B, so as to increase the response to P alone. Temperature 5° C.

Several other experiments of a similar kind were made; in some the result differed from that shown in Fig. 8, in that the Mg pulse reduced the response slightly even when it was applied immediately after P. It is not certain whether this was attributable to the magnesium ion, or to the small suppressing effect which a hyperpolarizing pulse had occasionally been found to produce (Katz & Miledi, 1967b). Apart from this small and inconsistent effect, the results were in line with those of Fig. 8.

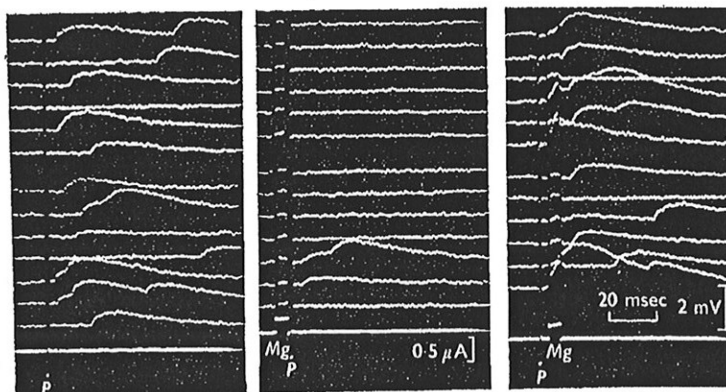

Fig. 7. Effect of *Mg*-pulses on end-plate response. Left column: Responses to depolarizing pulses *P* alone. Middle column: *Mg* pulse precedes *P*. Right column: *Mg* pulse follows *P*. Note: in the right column, the positive-going *Mg* pulse overlapped in time with the rising phase of some of the responses, and the associated focal hyperpolarization caused a transient increase in the amplitude of the e.p.p. (see Katz & Miledi, 1965*a*). Temperature 4° C. In this figure and Fig. 8, a *Ca*-pulse was applied in all trials, preceding *P* by about 15 msec, to raise the level of response sufficiently for inhibition to be demonstrable.

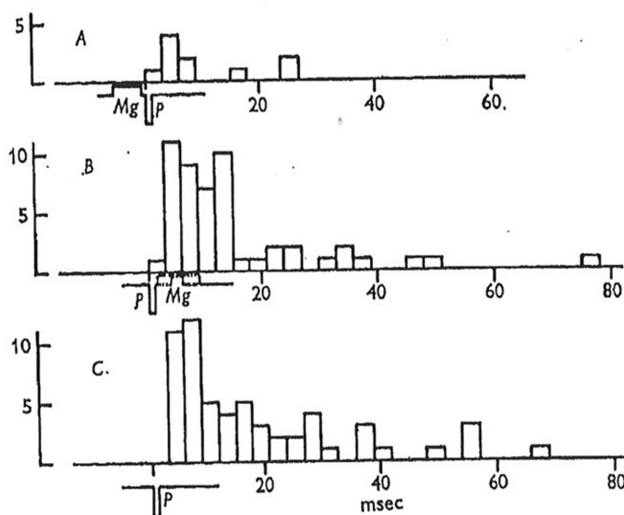

Fig. 8. Histograms of synaptic latencies. Abscissa: time interval between start of depolarizing pulse and start of unit e.p.p. Ordinate: number of observed unit potentials. Top histogram *A* shows distribution of responses to 61 (*Mg*+*P*) pulses. *Middle* (*B*): Responses to 65 (*P*+*Mg*) pulses. Two series with different *P*–*Mg* intervals as indicated by the interrupted and dotted outlines of the *Mg* pulses. Bottom histogram *C*: responses to eighty-three depolarizing pulses alone. Temperature 3° C.

DISCUSSION

It has been suggested that the first step by which depolarization of nerve endings leads to quantal release of acetylcholine is influx of calcium through the axon membrane (Hodgkin & Keynes, 1957; Katz & Miledi, 1967*b*). Recent observations have shown that there is a considerable delay between a brief depolarizing pulse and the release. The release may not even commence until sometime after the end of the depolarization (Katz & Miledi, 1965*b*, 1967*b*). The question, therefore, arises what happens during this latent period. Does the inward movement of calcium not begin until sometime after the end of the depolarizing pulse? Or is the delay largely due to subsequent reaction steps? In a recent paper (Katz & Miledi, 1967*b*), we suggested that calcium enters the membrane carrying net positive charge, either as Ca^{2+} or in the form of a compound CaR^{+}. This view was put forward to explain the peculiar 'latency shift', that is an increase in the minimum latency when the depolarizing pulse was lengthened.

The present findings suggest that the postulated entry of external calcium ions into the axon membrane is halted very soon after the end of the depolarizing pulse. Calcium ions which are made available on the outside after that brief initial period, have no influence on the process which has been set in motion by the depolarization. It may be that the opening of the external membrane 'gates' to Ca^{2+} or CaR^{+} is a transient event much briefer than the subsequent rise and fall of the probability of release. Or some calcium 'carrier' or 'receptor' only appears for a brief initial interval of time on the external surface of the axon membrane.

It will have been noted that the present technique is capable of resolving the time course of the calcium action with only limited accuracy. One may conclude that 'acceptance' of external calcium is terminated before the actual transmitter release commences. We cannot, however, distinguish between the period of depolarization and the latent period which immediately follows it. To do this, a method would have to be devised which enables one to reduce diffusion time even further than the present iono-phoretic pulse technique.

In summary, the most likely picture on the present evidence is this: (i) depolarization of the axon terminal opens a 'gate' to calcium; (ii) calcium moves to the inside of the axon membrane and (iii) becomes involved in a reaction which causes the rate of transmitter release to increase and which contributes a large part of the synaptic delay.

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