The release of acetylcholine from nerve endings by graded electric pulses

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(*Received* 29 *June* 1966)

1. The effect of brief depolarizations focally applied to a motor nerve ending was studied. Particular attention was paid to the relation between (i) strength and duration of the pulse and (ii) the size and latency of the resulting end-plate potential.

2. The release of acetylcholine lags behind the depolarization which causes it. If pulses of less than 4 ms duration are used (at 5 °C), the release starts after the end of the pulse.

3. Within a certain range, lengthening the pulse increases the *rate* of the ensuing transmitter release.

4. Unexpectedly, lengthening the depolarizing pulse also increases the latency of the transmitter release. This finding is discussed in detail. It is regarded as evidence suggesting that entry into the axon membrane of a positively charged substance (external Ca^{2+} ions or a calcium compound CaR^{+}) is the first step leading to the release of acetylcholine packets from the terminal.

Introduction

It has been shown in the preceding paper that the use of tetrodotoxin enables one to eliminate the nerve impulse and to study the process of transmitter release by applying electric pulses of graded strength and duration to the motor nerve ending. In the present paper this method will be employed to study certain quantitative aspects in more detail, in particular to examine the relation between the parameters of the applied pulse and the latency and size of the evoked response.

It had previously been found (Katz & Miledi 1965*b*) that there is an appreciable delay between the rise of the action potential wave in the nerve terminal and the release of acetylcholine. The cause of this delay is unknown, and it is of interest to find out whether this latent period can be altered by varying the intensity or duration of the imposed depolarizing pulse.

Methods

Much of the technical detail has been described in previous papers (Katz & Miledi 1965*a*, *e*). For most of the present work the focal method of pulse application (Katz & Miledi 1967*b*, method *B*) was preferred. By placing a microelectrode (a capillary usually of 2 to 4 μm tip diameter, filled with 0·5 or 3 M NaCl) over a junctional area and applying negative-going pulses to the pipette, a minute part of the junction could be depolarized for defined brief periods.

The pulse applied in this way alters the potential of a small surface of both the axon terminal and the underlying muscle fibre. This is represented schematically in figure 1. Suppose a 2 μm length of nerve ending of 1·5 μm diameter is covered by the micropipette and becomes uniformly depolarized by the applied surface-

negative pulse. The area of membrane which becomes discharged amounts to approximately 10 μm^2. The corresponding area of the muscle membrane underlying this part of the nerve ending is of the same order of size. The membrane potential change follows the applied current with very little time lag; a 'time constant' can be derived approximately from the product of the capacity of the small membrane area (about 10^{-7} μF) and the input resistance of the fibre which is of the order of 10^5 ohms for the muscle and 10^8 ohms for the nerve terminal. This would give 10^{-2} μs for the muscle and 10 μs for the nerve.

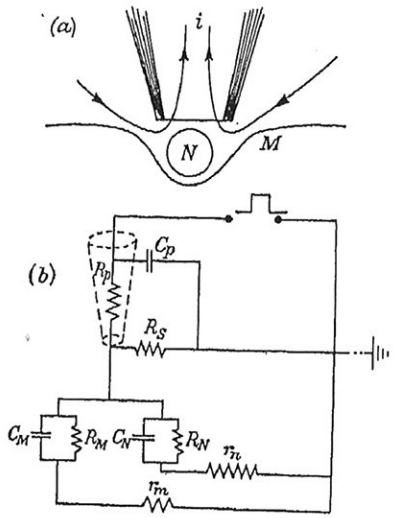


FIGURE 1. Diagram illustrating method of focal depolarization. (*a*) Tip of pipette placed over nerve-muscle junction. *N*, cross section of nerve terminal. *M*, muscle fibre. *i*, current entering tip of pipette. (*b*) Equivalent circuit, with tip of pipette indicated by dashed outline. C_p, capacity of glass wall of pipette; R_p, resistance of tip of pipette. R_s, shunt resistance on surface of nerve-muscle junction. C_m and R_m, capacity and resistance of localized surface of muscle fibre. C_N and R_N, capacity and resistance of local surface of nerve terminal. r_m and r_n, input resistances of muscle fibre and nerve terminal, respectively.

In the steady state most of the potential difference applied across R_s (i.e. between inside and outside of the pipette) will appear across the focal area of the membrane because the transverse resistance of this area is of the order of 10^{10} ohms, i.e. very much higher than the input resistance of the fibre. However, this ceases to be the case if the membrane resistance falls to a very low value or is actually caused to break down under the influence of an excessive electric pulse. This did, in fact, occur quite often, at least in the muscle fibre from which intracellular records were taken. It showed itself by the sudden appearance of an 'electrotonic potential', indicating that a low resistance had suddenly been established between the micropipette and the interior of the muscle fibre (see also Huxley & Taylor 1958). Evidence of local breakdown in the axon terminal was observed less frequently; it showed itself by a sudden prolonged outburst of miniature e.p.ps. When

these symptoms occurred the series of observations had to be discontinued and the pipette moved to another spot, or to another fibre.

The chief drawback of the method is that it does not provide adequate information on the amplitude of the pre-synaptic potential change. The best one can do is to obtain an indication of the *local displacement of the post-synaptic membrane potential* by the applied current. This is revealed by the change in size, and ultimately reversal of sign of the miniature e.p.ps which are elicited during the continued flow of the applied current. For example, suppose the membrane potential of the muscle fibre is -90 mV and the average unit e.p.p. (spontaneous, or occurring after a pulse) is 0·5 mV. The e.p.p. is known to reverse sign when the membrane potential is displaced by more than 75 mV, i.e. beyond -15 mV (del Castillo & Katz 1954*c*). Suppose a current of one particular strength elicits unit potentials of the same size, but opposite sign, then the membrane potential has presumably suffered a local displacement of 150 mV, from -90 to $+60$ mV. Rough estimates of this kind were made in several experiments and indicated that, post-synaptically at least, the shift of the membrane potential could reach an amplitude of several hundred millivolts before the local insulation of the membrane broke down. These calculations, however, do not provide a safe basis for estimating even approximately the change of the pre-synaptic membrane potential. Apart from the inaccuracy of this procedure, 'delayed rectification' may limit the focal displacement of the potential much more severely in the nerve ending than in the muscle fibre. This is because the input resistance of the axon terminal exceeds that of the muscle fibre by a few orders of magnitude. A lowering of the membrane resistance to, say, 10 ohm cm^2 would scarcely affect the distribution of potentials in the muscle fibre (between r_m and R_m; figure 1), but in the nerve ending this could cause the greater part of the potential drop to appear electrotonically across r_n.

A further difficulty is that a strong negative pulse from the pipette might conceivably subject the surrounding parts of the nerve terminal to a strong electrotonic hyperpolarization and lead to the 'anodal breakdown' effect, i.e. bursts of miniature e.p.p. (del Castillo & Katz 1954*b*; Katz & Miledi 1965*a*), originating *outside* the focal area. Such an event, however, would be easily recognized (*a*) from the abrupt appearance of bursts and long after-discharges following the pulse, (*b*) from the fact that the size and sign of such extra-focal discharges would *not* be altered by prolongation of the applied current. In our experience, with electrodes of 2 μm or larger diameter, the upper limit for the usable current strength was usually set by the occurrence of *post*-synaptic breakdown.

The upshot of these technical complications was that current intensities had to be kept within a limited range in which repeatable and consistent responses were obtained, and that only those problems could be studied which did not depend on an exact knowledge of the amplitude of the pre-synaptic potential change. The frequency of focal stimulation was usually kept low, with intervals between pulses ranging from 1·5 to 10 s. Even at these rates, e.p.p. responses tended to decline, and the statistical occurrence of failures to increase progressively. This was especially marked when the initial failure rate was low (and the value of m greater

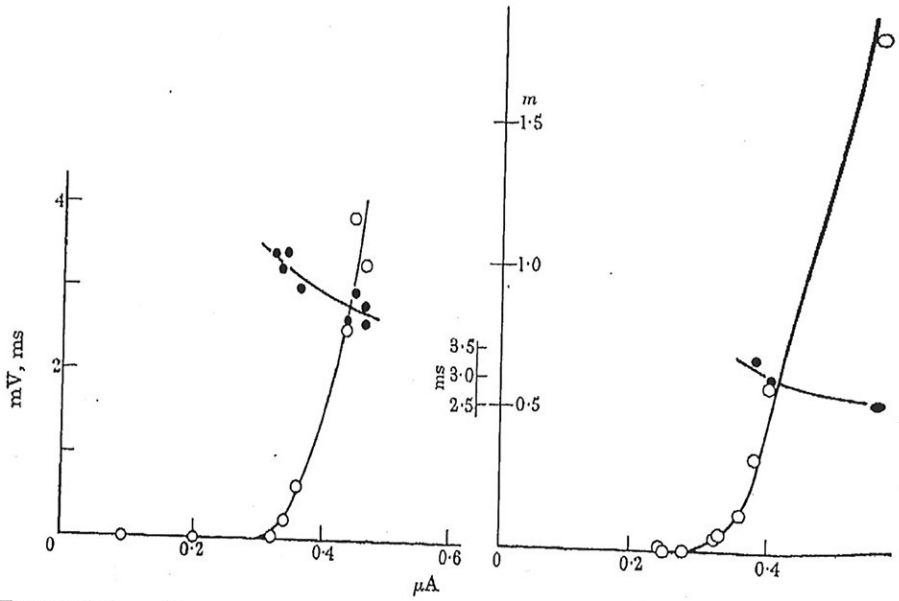


FIGURE 2. Strength-response relations obtained with focal, 1 ms pulses. Temp. 3·5 °C. In the series on the left, hollow circles show average amplitudes, in mV, of evoked e.p.p. On the right (from another series): hollow circles show mean 'quantal content' of evoked response, calculated from the proportion of failures. The mean unit size was approx. 1 mV in both series. Full circles: minimum latencies, in ms, measured from the start of the applied pulse to the beginning of the earliest e.p.p. Note: results shown in all figures were obtained by intracellular recording from muscles paralysed by tetrodotoxin (10^{-6} g/ml.).

than a small fraction of unity). This is not very surprising, for an average release of one unit per pulse from a few microns length of nerve terminal corresponds to nearly the full rate of release by a normal nerve impulse.

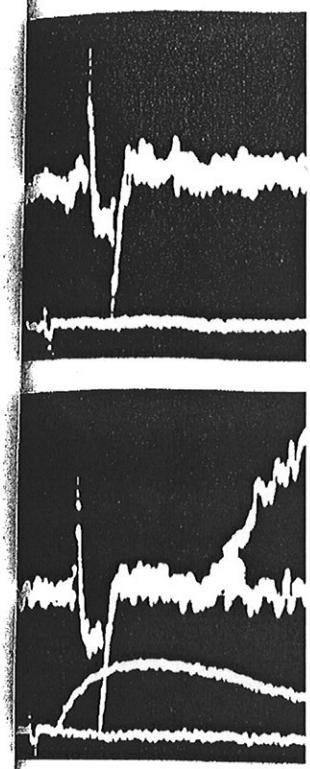
Recording

The response was recorded intracellularly from the muscle fibre, with a suitably placed external microelectrode serving as reference lead. The residual artifacts associated with the applied pulse were not sufficiently large to obscure the time course of the evoked e.p.p. The 'artifact' included a small electrotonic potential change in the muscle fibre which arose from the 'convergence field' set up in the Ringer's fluid surrounding the polarizing micropipette. This amounted usually to less than 1 mV, well below 1% of the focally applied potential change. As before (Katz & Miledi 1967*b*) prostigmine was used in most experiments.

RESULTS

The effect of strength and duration of focally depolarizing pulses on the size of the end-plate response

If one increases the intensity of a short pulse of constant duration, the response rises in a non-linear manner as shown in figure 2. The behaviour resembles the



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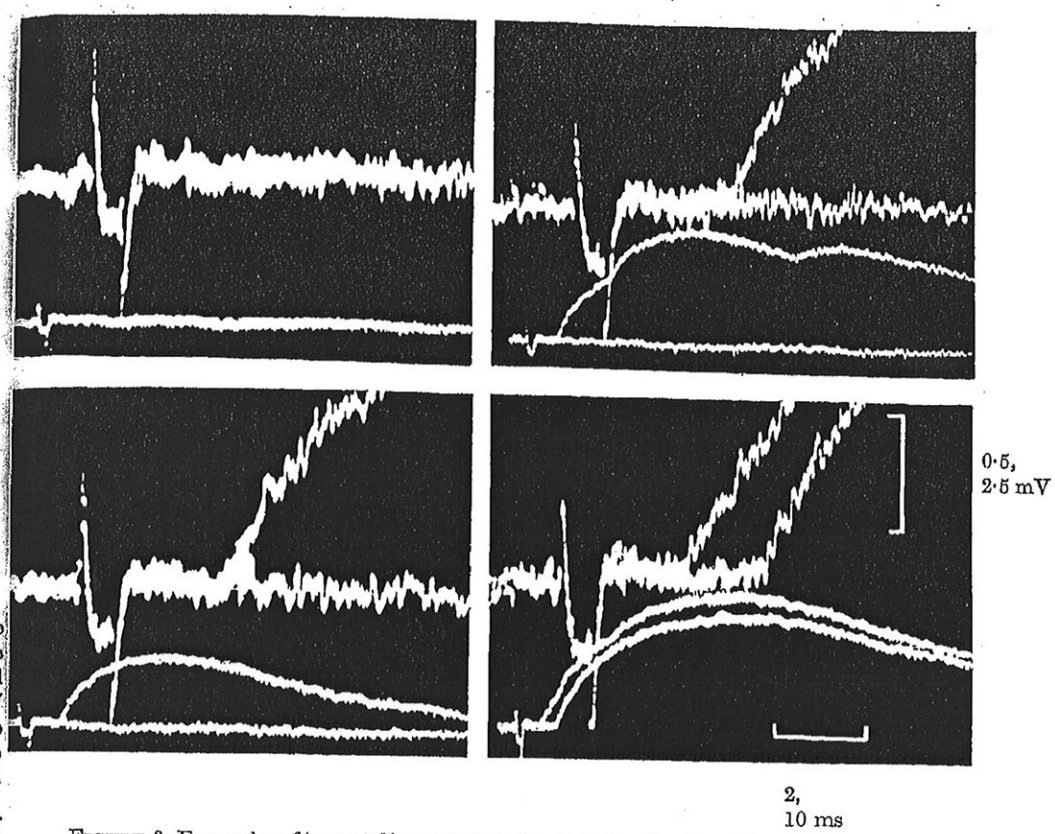

FIGURE 3. Examples of 'quantal' e.p.ps evoked by brief focal pulses. Temp. 4·5 °C. Each block shows two simultaneous recordings, the top record at the higher speed and amplification indicated on the scales. Each recording consists of two superimposed traces. The column on the left was obtained with a slightly weaker pulse. Note: 'all-or-none' appearance of discrete units of response, and variable latencies.

effect already described with the method of electrotonic application. Below a certain intensity, the probability of observing even a single unit discharge is very small; above this apparent threshold, occasional unit e.p.ps are seen (figure 3), and a further increase of current strength causes a steep rise in the amplitude, i.e. the number of quantal units, of the e.p.p. (see Katz & Miledi 1967*b*).

A similar non-linear increase in the size of the e.p.p. is observed if one increases the duration of a pulse of constant intensity from, say, 0·2 ms to a few milliseconds (figure 4).

If one determines the 'threshold' intensity of pulses of different durations, taking the appearance of an occasional unit e.p.p. as threshold index, one obtains a 'strength duration' curve of the kind shown in figure 5 (cf. Katz & Miledi 1965*d*). This has the usual hyperbolic shape indicating that for very brief pulses, the transmitter release depends on the time integral of the depolarization rather than on its amplitude.

The interpretation of this curve differs from that of the *conventional* type of strength-duration relation which is related to the electric time factor of the membrane (see Katz 1939). In the latter case depolarization rises gradually during a current pulse; the shorter the current, the greater must be its strength if

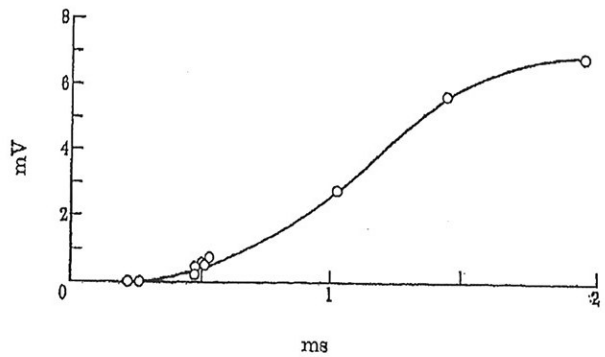

FIGURE 4. Effect of pulse duration on size of evoked response. Temp. 7 °C. Ordinate: average amplitude of evoked e.p.p. Abscissa: duration of applied pulse. Pulse intensity was 3·25 μA.

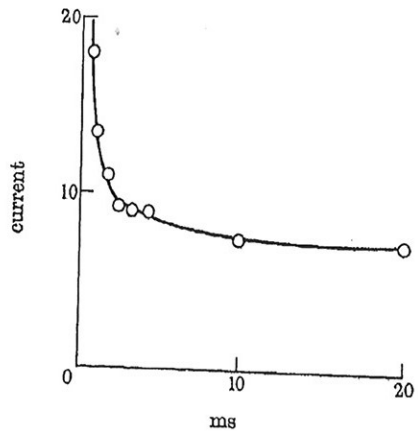

FIGURE 5. 'Strength-duration' curve, showing the relation between duration and intensity of equally effective pulses. Temp. 9·5 °C. The duration (abscissa) was varied, and the current strength (ordinate, relative units) was determined which caused occasional unit responses to appear at the end of the pulse. The 'rheobase' (intensity required for 20 ms pulse) was about 0·15 μA.

a given potential change is to be attained. In the present experiments, in which a focal microelectrode is used, the electric time factor is probably much less than 0·1 ms (see Methods), and the pulses of focal depolarization produced by the currents in figures 4 and 5 were presumably 'square' except for a small fraction of a millisecond at beginning and end.

The disproportionate increase in the response with increasing pulse duration (figure 4) indicates that the *rate* of transmitter release which results from a

depolarization of given amplitude, is not determined by that amplitude alone but continues to rise when the depolarization is prolonged up to several milliseconds in duration. Further examples are shown in figure 9 where an increase in pulse length from 0·5 to 2·12 ms caused the average quantal release m (del Castillo & Katz 1954*a*) to increase from 0·07 to 0·97 units per pulse, in spite of a falling pulse intensity. The value of m increased roughly as the square of the Coulomb quantity of the pulse.

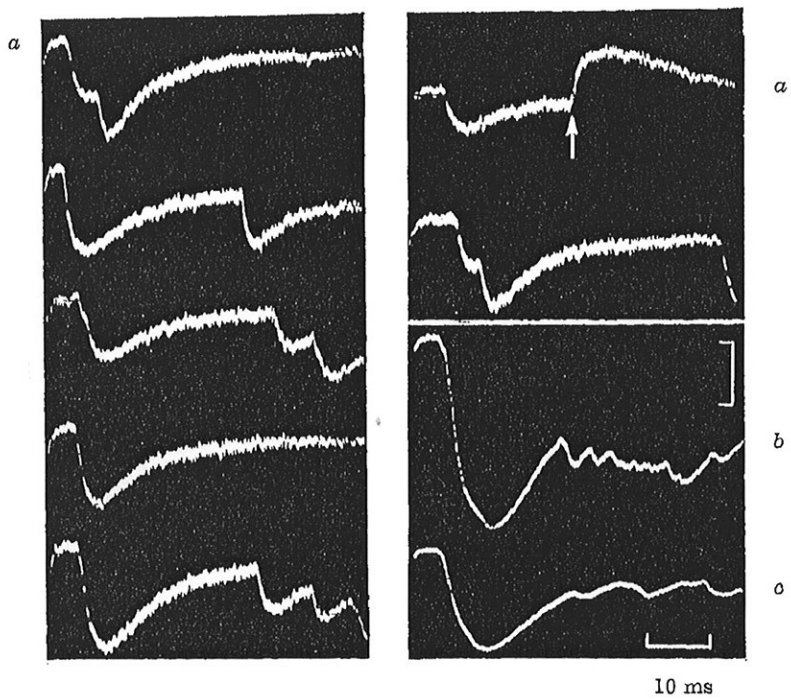

FIGURE 6. Inverted e.p.ps (i.e. negative-going potentials with intracellular recording) *during* focal current application. Temp. 7 °C. Current strength approx. 0·7 μA (varied between 0·57 and 0·76 μA). Duration of pulse 50 ms, starting at beginning of trace. Vertical scale 0·4 mV in *a*, 1 mV in *b*, 2 mV in *c*. Current strength was raised in *b* and *c*. About 150 pulses were given at 5 s intervals. Throughout the series, only one potential (at arrow) had the normal, positive-going, sign; this was presumably a spontaneous min. e.p.p. arising outside the focal depolarized zone.

This applies to *brief* periods of depolarization, say up to 2 or 3 ms at 5 °C. If the duration of the current is lengthened further, the rate of release, measured as the frequency of evoked miniature e.p.ps, undergoes a complex sequence of changes, an example of which is shown in figures 6 and 7. These figures illustrate the temporal distribution of miniature e.p.ps recorded during a series of strong depolarizing pulses of 50 ms duration. Samples of the records from which the histograms in figure 7 were compiled are shown in figure 6. It will be noted that these potential changes are of opposite electric sign to spontaneous potentials and to those recorded after the end of a pulse (e.g. figure 12), for reasons already explained (Methods and

Katz & Miledi 1965*a*). The size of the early peak in the histograms must be an underestimate because, during 'peak times', units were crowded together into nearly synchronous reponses, and not all their miniature components could be resolved. The general features, however, are clear, viz. that during the large depolarizing current the frequency of quantal transmitter release rises after a brief delay and builds up to a peak in several milliseconds, from which it falls to a low level. This is followed by a slow secondary rise. A detailed investigation of these

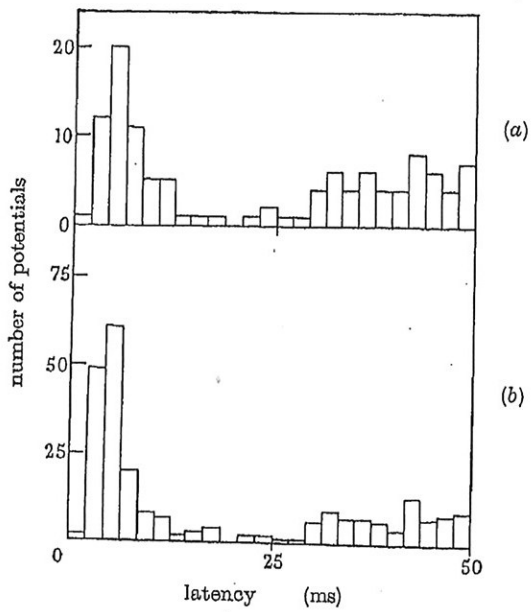

FIGURE 7. Latency histogram of e.p.ps from the experiment illustrated in figure 10. (*a*): Response during the first 50 pulses. (*b*): Histogram of the whole series (145 pulses). There was a gradual decline in the *later* part of the response.

complex changes which are presumably related to the 'refractoriness' and 'facilitation' described in the previous paper must be left to a later study. In some experiments, the phase of depression shown in figure 7 was not evident; this may well depend on such variable factors as the size of the focal pipette and the relative resistances of the nerve ending and its depolarized membrane area (see p. 25).

The present observations indicate that one of the assumptions which was basic to Liley's (1956) hypothesis, namely that the rate of transmitter release is determined by the value of the pre-synaptic membrane potential at that instant, is not valid. The reason why the striking time-dependence of the process has not previously been recognized lies in the facts that earlier experiments were made at a high temperature at which transient phenomena are confined to a much shorter initial period, and that in the absence of tetrodotoxin the currents had to be established gradually to avoid initiation of impulses. This procedure inevitably obscures the phenomena which have been reported here.

The delay between depolarization and quantal release

The results so far described show the relations between size and duration of the depolarizing pulse and the resulting *intensity* of transmitter release. In the present section, the *time course* of the resulting transmitter release will be considered.

The main observations are presented in figures 11 and 12 and the histograms of figures 8 to 10. They contain two important features. First, unit e.p.ps do not begin to appear until well after the end of the depolarizing pulse, provided the duration of this pulse does not exceed a few milliseconds. It was previously shown (Katz & Miledi 1965*b*) that the beginning of the unit e.p.p. indicates, approximately, the moment at which a quantal packet of acetylcholine is released. The

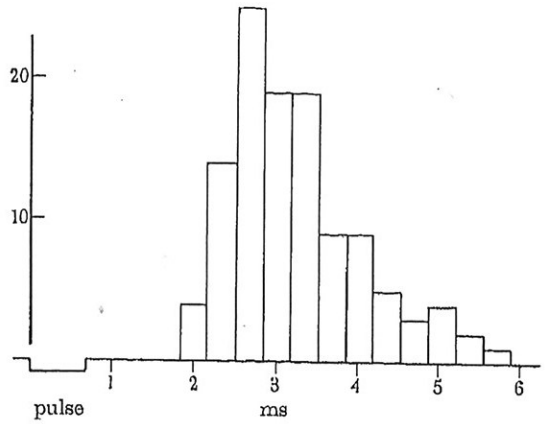

FIGURE 8. Latency distribution of e.p.ps evoked by constant, focal current pulses of 0·68 ms duration. Temp. 4·5 °C. Ordinate: number of observed responses. Abscissa: time interval between start of depolarizing pulse and beginning of e.p.p.

error incurred in assuming coincidence of these two events is probably no more than 0·1 ms (Katz & Miledi 1965*e*). It seems safe to conclude that, with these brief focal pulses of depolarization, the resulting transmitter release does not start until some 1 to 2 ms have elapsed after the depolarization has subsided. This is quite unlike the release of potassium ions (Hodgkin & Huxley 1952) which attains its maximum intensity *during* the depolarization. These observations suggest that there is likely to be a series of intermediate reactions between the membrane potential change and the release of acetycholine.

The second point which deserves attention is the latency shift which was consistently observed when a pulse of given strength was altered in duration.

The 'latency shift'

When the pulse is lengthened, e.g. from 0·5 to 2 ms, the process of transmitter release is influenced in two apparently opposite ways. There is the very large increase in the total amount released which has already been described. Unexpectedly, however, the delay—measured from the start of the depolarizing pulse—is *lengthened*. This effect is small, but consistent. Examples are shown in figures 9 to 12 and table 1.

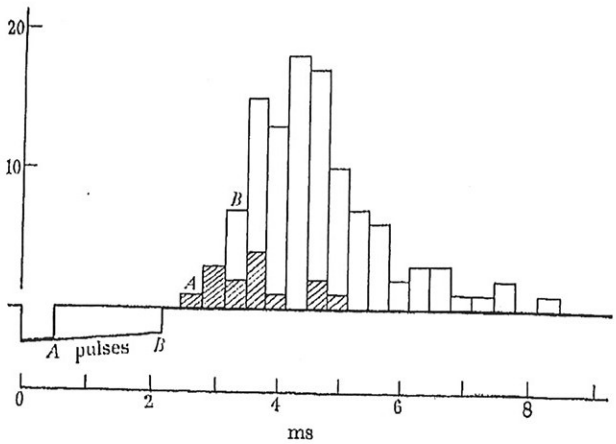

FIGURE 9. Effect of pulse duration on the latency histogram. Two pulses (*A* and *B*) were applied. The latencies of evoked e.p.ps are shown, respectively, by shaded and clear columns. Temp. 4·4 °C. The short pulses (*A*) evoked only 14 e.p.ps in 211 trials; hence $m = \ln 211/197 = 0{\cdot}07$. The longer pulses (*B*) produced 104 responses in 168 trials; hence $m = \ln 168/64 = 0{\cdot}97$.

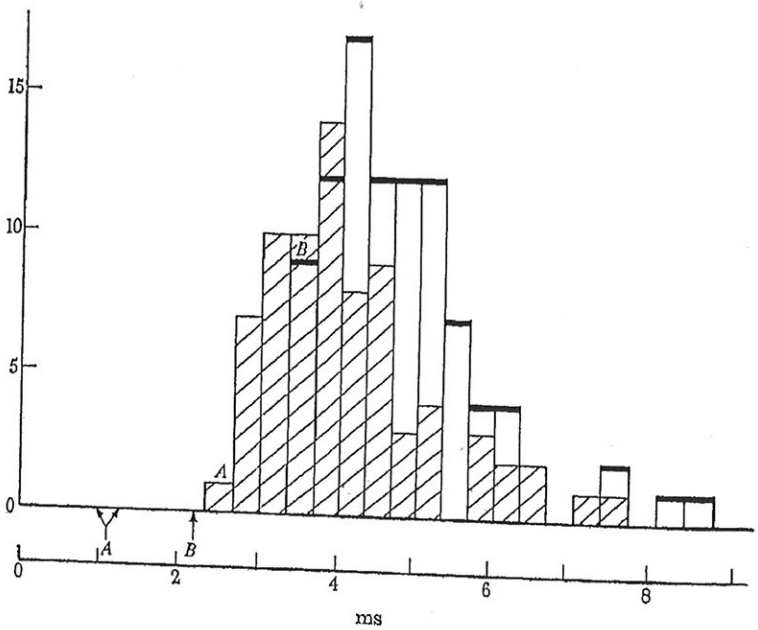

FIGURE 10. Another example of the 'minimum-latency shift' caused by a change in pulse duration at constant pulse intensity. Durations of pulses are indicated by arrows at *A* and *B*. The responses to *A* (424 pulses) are shown by shaded columns, those to *B* (166 pulses) by heavy crossbars (and, where numbers of *B* responses exceed those of *A*, by clear rectangles). Temp. 5 °C.

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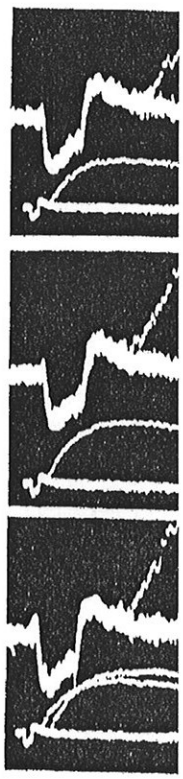

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If one measures the delay from the *end* of the pulse, this diminishes with increasing pulse duration, and eventually the e.p.p. is seen to start during the pulse itself. If the focally applied current is strong, the e.p.p. may be inverted while the pulse lasts (figure 12), but reverts to the normal polarity as soon as the pulse is withdrawn.

The absolute values of the latencies depend not only on pulse duration, but also on its intensity (figure 2) and on the temperature. At 5 °C, the shortest delays

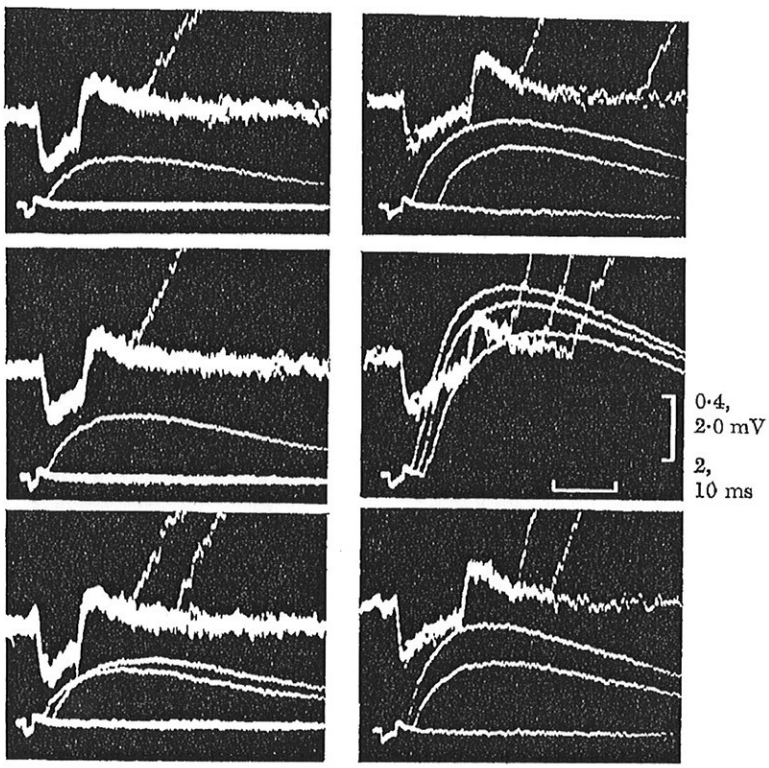

FIGURE 11. Sample records from the experiment illustrated in figure 10. Left column: short pulses (*A*, figure 10); right column; long pulses (*B*). Simultaneous recordings in each block at two speeds and amplifications. Each recording on the left consists of 6, on the right of 3, superimposed traces.

observed with strong *brief* pulses were 1·5 to 2 ms, the earliest e.p.p. observed during a strong *long*-continued current started after 4 to 5 ms.

The fact that a prolongation of the depolarizing pulse not only increases transmitter output, but delays its appearance, provides an interesting clue to the physico-chemical mechanism of release and will be discussed in detail below.

An obvious further experiment was to apply pulses of opposite sign during the latent period, in order to see whether hyperpolarization immediately after the depolarizing pulse would cancel, or at least modify, the transmitter release. The results of this type of experiment, however, were not very revealing. The procedure

was to use depolarizing pulses (P), of 0·4 to 2 ms. duration, and follow them after a brief interval (20 to 140 μs) with 'anodic' pulses (A) of the same or somewhat lower intensity. Several series of observations were made; to summarize, the anodic pulses were either ineffective or produced a small reduction in transmitter output, without obvious change in the latency distribution. With very brief intervals between P and A pulses, the suppressing effect of the hyperpolarization, though small, was statistically significant. Thus, with approximately 20 μs separation

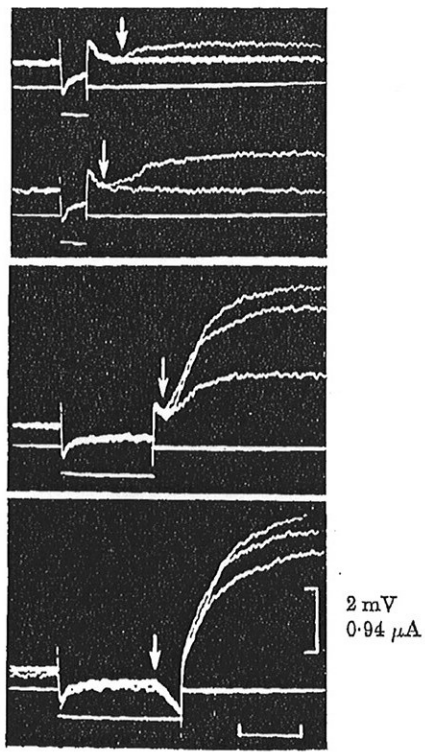

FIGURE 12. Further example of 'latency shift'. Three pulse durations. Beginning of e.p.ps shown by arrows. Note: inversion of e.p.p. *during* long current pulse (bottom record). Temp. 4·5 °C. The preparation was in a medium of low sodium and calcium content (half sodium replaced by sucrose, 0·2 mM Ca).

257 P pulses evoked 100 responses. The same number of combined $P+A$ pulses produced only 74 responses. When intervals of 115 μs were used, the proportion of responses was 139/419 and 118/400, to P and $P+A$ respectively, a difference which does not pass the usual tests of statistical significance. Similar results were obtained in another experiment, where at 45 μs separation the depolarizing pulses alone evoked 154 responses in 417 trials, while $P+A$ gave 59 responses to 243 pulses. When the intervals were lengthened to 140 μs, no difference between P and $P+A$ effects was seen. Thus, it appears that the anodic pulses given during the

latent period are not very effective in interfering with the release mechanism. This contrasts with the powerful anodic suppression described in a previous paper (Katz & Miledi 1967*a*) where the hyperpolarizing pulse was applied immediately after the negative peak of the focal axon spike, i.e. during the decline of the membrane action potential. The difference presumably arises from the fact that, in the present case, there is no appreciable *direct* interference between opposite membrane potential changes, the anodic pulse being applied *after* the depolarization, while in the previous case the anodic pulse overlapped with and curtailed the action potential, and so greatly reduced its efficacy in releasing transmitter.

TABLE 1. THE 'LATENCY' SHIFT

Results were from a single end-plate at about 5·2 °C; observations with 1·0, 1·27 and 2·2 ms pulses were made at one spot.

pulse duration (ms)	number of applied pulses	number of responses	minimum latency (ms)
0·5	23	3	2·1
1·0	98	14	2·4
1·27	326	61	2·7
2·2	166	96	3·4

Triggered 'giant' responses

Under certain abnormal conditions, the graded relation between the size of the pulse and of the evoked e.p.p. is replaced by an entirely different phenomenon, viz. a huge, almost explosive release which is triggered suddenly at an unpredictable pulse strength. This was observed in an experiment in which 5 mM tetraethylammonium bromide had been added (figures 13 and 14). The response consisted of an e.p.p. of about 50 mV which subsided slowly and showed fluctuating high-frequency components of multiple miniature e.p.ps during its decline. A similar effect was seen in two experiments in which a dose of tubocurarine had been given together with the tetrodotoxin, and the nerve was subjected to a series of very strong depolarizing pulses.

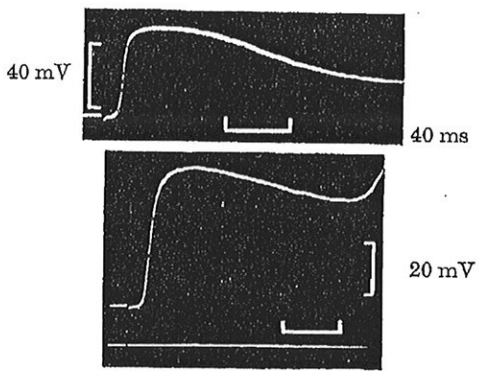

FIGURE 13. Triggered 'giant' e.p.ps Preparation was in a solution containing 5 mM tetraethylammonium bromide. Temp. 3·5 °C. Pulse was applied to motor axon ('electrotonic' method).

The quantal content of the enormous e.p.ps shown in figure 13 must have been well over one thousand, judging from the relatively small sizes of the spontaneous miniature potentials (about 0·15 mV). The mechanism by which these huge responses are triggered is obscure. It was noted that they occurred during a transient state of abnormal instability, which was characterized by a high frequency of spontaneous discharge and which was followed by total failure of any evoked response.

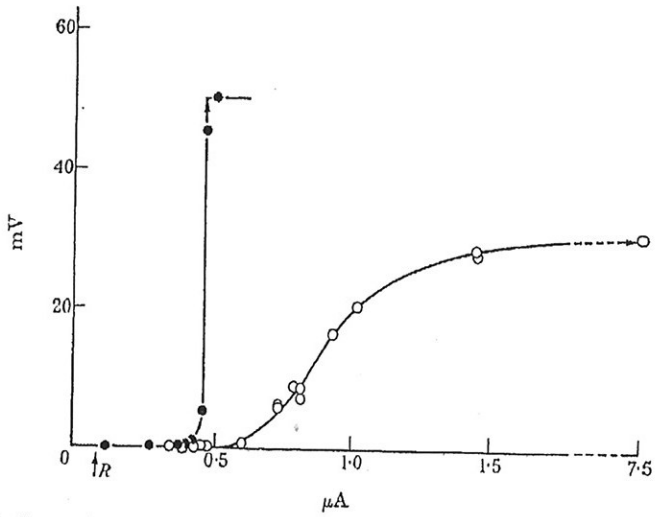

FIGURE 14. Strength-response relations, showing amplitude of e.p.ps (ordinate) evoked by 5·5 ms current pulses applied to the motor axon (method *A*, Katz & Miledi 1967*b*). Abscissa, current strength. Hollow circles: before adding 5 mM tetraethylammonium. Full circles: after adding *T.E.A.*. *R*: 'rheobase' determined at beginning of experiment (before applying tetrodotoxin).

DISCUSSION

The central piece of evidence presented in this paper is contained in figures 9 to 12. These show (*a*) that the release of the transmitter is delayed and, in fact, only starts after the end of the brief depolarization which initiated it, (*b*) that lengthening the depolarizing pulse causes a larger amount to be released *after a longer delay*.

In searching for a reasonable interpretation of these findings, it should be recalled that external calcium ions are essential for the depolarization to become effective (Katz & Miledi 1965*c*).

We envisage that the evoked release of quantal packets of acetylcholine proceeds in several stages the first of which is an inward movement through the axon membrane either of Ca^{2+} itself, or of a positively charged calcium compound CaR^{+}. The subsequent steps could be pictured in various ways, e.g. according to the vesicular hypothesis as a reaction on the inside of the membrane by which Ca or CaR causes momentary fusion of vesicular and axon envelopes at their point of collision (del Castillo & Katz 1957; Katz 1962).

We are concerned here only with the first link of this chain. The surface-negative pulse has two opposite effects: it facilitates this process by raising the

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membrane 'permeability' to Ca^{2+} or CaR^{+} ions; but the negative pulses also oppose the inward movement of the positive ions. The former reaction presumably develops and decays with a time lag, while the latter is a direct electrophoretic effect which might well account for the delaying action of the applied current.

On the basis of this hypothesis, one might expect that a sufficiently large negative surface potential would completely prevent inward movement of the postulated Ca-compound, and that transmitter release could in principle be delayed until the end of the pulse regardless of its duration. This was not observed: the delay was limited to 4 to 5 ms at 5 °C, even in an experiment in which the external calcium concentration had been reduced to about 0·1 mM. However, this finding presents no decisive argument against our hypothesis because the relevant quantities (amplitude of the pre-synaptic potential change, concentration difference and 'equilibrium potential' for the transfer of Ca^{2+} or CaR^{+}) are unknown.

The minimum latencies obtained with intense and very brief pulses are shorter than the minimum 'synaptic delay' between nerve spike and e.p.p. at the same temperature (Katz & Miledi 1965*e*). For example, at 5 °C, a strong pulse of 0·5 ms duration can elicit a unit response with a latency of 1·5 to 2 ms as compared with 2 to 4 ms minimum spike-e.p.p. delay. There are two probable causes of this difference: (*a*) the current pulse was of very high intensity, close to the strength at which postsynaptic breakdown tends to occur (see Methods). Hence the brief local displacement of the membrane potential of the nerve may have greatly exceeded that attained during the normal impulse; (*b*) the duration of the nerve action potential is longer than the 0·5 ms pulse, and that would also shift the latency in the observed direction. It is indeed possible that the large temperature coefficient of the synaptic delay results in part from the large effect of temperature on the falling phase of the action potential, a correlation to which attention has already been drawn in a previous paper (Katz & Miledi 1965*e*).

The 'strength-response' relations shown in figure 2 are of interest in that they help one to assess the relative power of the nerve impulse in releasing transmitter. Only a portion of the focal 'strength-response' relation was obtained, because the usable range of intensities is limited by the current strength at which the muscle membrane breaks down (see p. 24). The length of nerve subjected to the stimulus was of the order of a few microns, approximately 1% of the total length of the junctional branches. Normally (with a calcium concentration of 1·8 mM), a nerve impulse causes the release of about 200 quantal units. In the experiments of figure 2, the size of the unit potentials was approx. 1 mV; the 1 ms pulses were able to release 2 to 4 units from the small area under the electrode, and there was no sign of a 'ceiling' being reached. Moreover, the duration of the pulse was shorter than that of a nerve spike at 3·5 °C. It appears from this experiment that the 'secretory power' of the nerve impulse is well below that of a 'maximum stimulus' though it might approach saturation in high calcium, or during tetanic potentiation.

That the release of transmitter increases more than in direct proportion to the imposed depolarization is known from previous work (del Castillo & Katz 1954*b*; Liley 1956; Takeuchi & Takeuchi 1962; Miledi & Slater 1966). It was not known, however, that there is a similar non-linear increase when the *duration* of the

depolarization is lengthened (figure 4). This observation must affect any analysis of the release evoked by a nerve impulse. If one visualizes a depolarization of a few milliseconds duration as being made up of a series of much briefer pulses, then each of them apparently facilitates, or 'potentiates', the effect of its successor. The basis of this process of facilitation is not known. It might be due to accumulation of calcium ions or a calcium compound at the inside of the axon membrane (cf. Katz & Miledi 1965*c*; Gage & Hubbard 1966), an idea which would also fit with recent observations by F. A. Dodge & R. Rahamimoff (unpublished), that transmitter release increases much more than in direct proportion to the external calcium concentration.

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