J. Physiol. (1969), **203**, *pp.* 689–706
With 14 *text-figures*
Printed in Great Britain

SPONTANEOUS AND EVOKED ACTIVITY OF MOTOR NERVE ENDINGS IN CALCIUM RINGER

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(*Received* 2 *April* 1969)

SUMMARY

1. Electrical activity of neuromuscular junctions of the frog was studied in a medium (Ca-Ringer) whose Na ions had been entirely replaced by Ca.

2. Spontaneous miniature end-plate potentials (m.e.p.p.s) of reduced amplitude are recorded in this abnormal ionic environment, and graded end-plate potentials can be elicited by applying depolarizing current pulses to the pre-junctional parts of the nerve.

3. Addition of 5 mM tetraethylammonium (TEA) to the Ca-Ringer causes the appearance, in almost all-or-none fashion, of very large e.p.p.s (up to 45 mV in amplitude) in response to nerve stimulation.

4. These 'giant' e.p.p.s occur despite the curarizing action of TEA (and its depressing effect on the amplitude of m.e.p.p.s) and they persist after application of tetrodotoxin.

5. After several hours exposure to Ca-Ringer, spontaneous end-plate activity gradually declines, and eventually evoked e.p.p. responses fail. On return to normal Na-Ringer, spontaneous end-plate activity is quickly resumed, but the potentials have an abnormal, very wide, amplitude distribution.

6. The results are discussed, in conjunction with relevant work on the squid giant synapse, in terms of the 'calcium hypothesis' of transmitter release.

INTRODUCTION

Takeuchi (1963) has shown that frog muscles placed in an isotonic solution of $CaCl_2$ remain viable and have good resting potentials, and that the junctional regions of the muscle fibres respond with measurable depolarization and local contraction to applied acetylcholine (ACh). These observations make it possible to find out whether ACh can still be released from the nerve endings when all the external Na has been replaced by Ca.

In a brief note (Katz & Miledi, 1967*e*), it was shown that both spontaneous miniature end-plate potentials (m.e.p.p.s) and evoked e.p.p.s can still be obtained under these conditions, confirming the view that external Na is not immediately required for the release of acetylcholine. In the present paper, these experiments are described more fully; in addition it will be shown that the graded form of transmitter release, due to applied current pulses, can be converted into a triggered, 'explosive' type of response by adding several mM-TEA to the isotonic Ca-Ringer. This is of interest because, in conjunction with previous work (Katz & Miledi, 1967*c*, *d*, 1969) it indicates that Ca influx into presynaptic terminals can under special conditions provide sufficient inward current to produce a regenerative response.

METHODS

The experiments were made on myoneural junctions of frog sartorius muscles, during June 1967–July 1968. Unless otherwise stated, the muscles were immersed in a solution containing 83 mM-$CaCl_2$, 2 mM-KCl and, usually, neostigmine methylsulphate, 10^{-6} g/ml. In this solution, frog nerve and muscle fibres do not give action potentials, and it was therefore unnecessary to use tetrodotoxin (TTX) when studying the effects of graded depolarization. Most experiments were made at low temperature (4–11° C). Recording was by intracellular, KCl-filled micro-electrodes, the reference electrode in the bath being a chlorided silver wire. To depolarize motor nerve endings, two methods previously described (Katz & Miledi, 1967*b*) were used. With one ('electrotonic') method, a small intramuscular nerve branch was isolated and followed by microdissection to a point close to a group of superficial junctions. The nerve was then lifted into a layer of liquid paraffin above the Ca-Ringer with the muscle close to the oil/water interface. The current pulses were applied through chlorided silver wires, one on the thick part of the sciatic nerve, the other in the muscle bath. In this way, the pre-terminal portions of the nerve fibres could be depolarized, and the potential change allowed to spread electrotonically along the whole of the terminal branchwork. With the second ('focal') method, localized parts of superficial myoneural junctions were depolarized by applying pulses through a micropipette, filled with 1 M-$CaCl_2$ and placed directly over a junctional region.

RESULTS

General observations. Preparations could be kept in isotonic Ca-Ringer at low temperature for several hours, without obvious deterioration. High and stable values of resting potentials (around 100 mV) were obtained, and it was noticed that, although microscopically visible damage sometimes occurred around the point of electrode insertion, this tended to 'heal' quickly and the local appearance of the fibre reverted to normal. With a second current-passing electrode graded local contractions could be elicited, but the threshold for the contractile response appeared to be much higher than in ordinary Ringer (+TTX). Depolarizations of over 70 mV, displacing the membrane potential to or beyond −30 mV (instead of about −50 mV) were needed.

Effect of calcium substitution on miniature potentials

When the recording electrode was inserted at a myoneural junction, spontaneous m.e.p.p.s could often be detected, though in some muscle fibres it was necessary to hyperpolarize the membrane with a second electrode in order to make the spontaneous potentials visible above noise

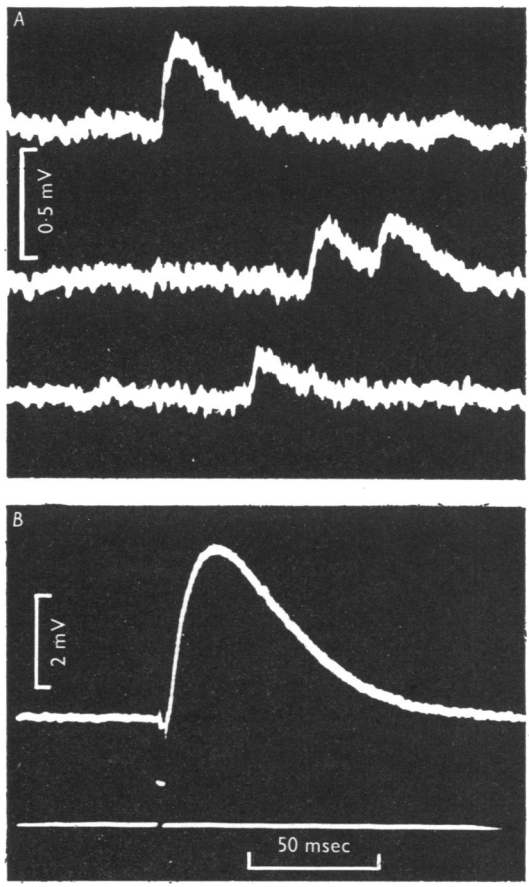

Fig. 1. End-plate activity in isotonic Ca. 6·5° C. Intracellular recording from junction of frog sartorius muscle. *A*: spontaneous m.e.p.p.s at resting potential 96 mV. *B*: e.p.p., evoked by brief, focal pulse (shown in bottom trace).

level. Examples of m.e.p.p.s in Ca-Ringer are shown in Figs. 1*A* and 14*A*. The records in Fig. 1 were obtained at a temperature of 6·5° C, 6 hr after the isotonic Ca solution had been introduced. In general, replacement of normal Ringer by isotonic Ca caused immediate reduction in the mean amplitudes of the spontaneous potentials to about 1/5–1/6 (cf. Table 1).

During the next several hours, the amplitudes remained fairly stable or diminished relatively little, while the frequency underwent a more complicated progressive change, first increasing and eventually declining well below the normal rate (see p. 702). The results in Table 2*A* were obtained during relatively short Ca exposure, before the rate of the discharge had reached its peak.

TABLE 1. Effect of isotonic Ca on size of m.e.p.p.s

Temp. 7·5° C.

Period in isotonic Ca: 90 min. Neostigmine present throughout.

Mean resting potentials: 76 mV in normal solution, 100 mV in isotonic $CaCl_2$.

	Mean amplitude of m.e.p.s in mV				
Fibre	Normal	Ca	Normal	Mean normal	Ratio normal/Ca
1	1·22	0·24	1·33	1·28	5·35
2	0·47	0·10	0·55	0·51	5·1
3	1·06	0·26	1·28	1·28	4·95
4	0·86	0·16	0·94	0·94	5·9
5	0·65	0·16	1·1	0·88	5·5
				Mean ratio	5·4

TABLE 2. Frequency (sec^{-1}) of m.e.p.p.s in the same experiment as Table 1

Fibre	Normal	Ca	Normal	Mean normal	Ratio Ca/ normal
1	0·177	0·535 (occasional bursts)	0·104	0·141	3·8
2	0·353	(0·395, too small)	0·2	0·277	(1·42)
3	0·184	0·575	0·124	0·154	3·7
4	0·21	0·83	0·177	0·199	4·2
5	0·091	0·345	0·035	0·063	5·5

Disregarding high-frequency bursts in fibres 1, 3 and 4 in isotonic Ca, and fibre 2 whose 'Ca' m.e.p.p.s were too small, this gives approx. four- to fivefold increase in frequency during the 90 min exposure to isotonic Ca.

In two experiments, the effects of substituting isotonic Mg, instead of Ca, were examined. The changes in the spontaneous activity were rather similar: there was an immediate reduction in mean amplitude of m.e.p.p.s (e.g. from 1·1 to 0·14 mV). The frequency was not affected immediately. However, after a delay (of about 4 hr at 5° C and less than 1 hr at 20° C), the rate of the discharge became very high in all fibres. The similarity of these effects of Mg and Ca on spontaneous activity stands in interesting contrast with their opposite actions on depolarization-evoked release.

It should also be mentioned that the 'sealing' of membrane damage observed in isotonic Ca, was not found in Mg-treated muscles whose resting potentials were, therefore, generally lower and less stable.

Calcium substitution and local neuromuscular transmission

Having shown that the spontaneous release of ACh packets continues in an isotonic Ca solution, the question remained whether it is possible (as in TTX-treated end-plates) to elicit e.p.p.s by local depolarization of the nerve terminals. The experiments described below were intended to give a

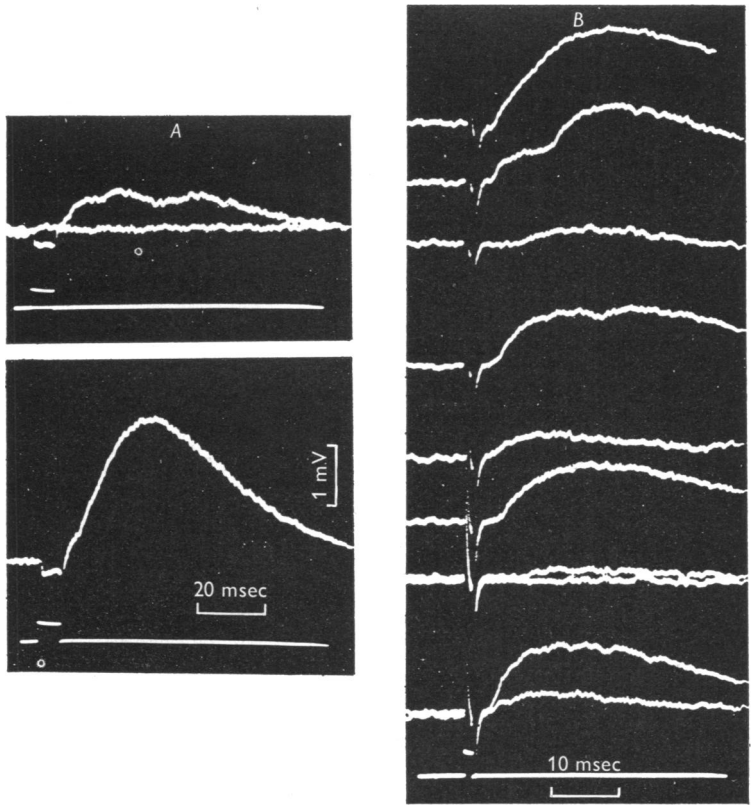

Fig. 2. E.p.p.s evoked by focal pulses in Ca-Ringer. Same experiment as Fig. 1. *A*: responses to two 7 msec pulses of different intensities. *B*: fluctuating responses to repeated pulses (1·3 msec duration, constant strength). The pulses are monitored in the bottom traces.

qualitative answer to this question; a quantitative comparison, between the efficacy of synaptic transfer in normal and Ca-substituted Ringer was not attempted, although it appeared that, in the latter, stronger currents were needed to obtain e.p.p.s.

Figure 1*B* shows an example of an e.p.p. evoked by focal depolarization of a motor terminal, after 6 hr exposure to the isotonic Ca solution. With weaker pulses, smaller fluctuating e.p.p.s were obtained, illustrated in

Fig. 2. The response is evidently made up of quantal steps whose numbers and latencies fluctuate during successive pulse applications. Figure 3, still from the same end-plate, shows a latency histogram from such a series. The average number of responding units was somewhat greater than 2; the histogram is, therefore, incomplete and, inevitably, many individual unit latencies have been missed. Nevertheless, the general distribution is clear and closely resembles the picture in a normal ionic environment (cf. Katz & Miledi, 1965*a*, *b*; 1967*c*).

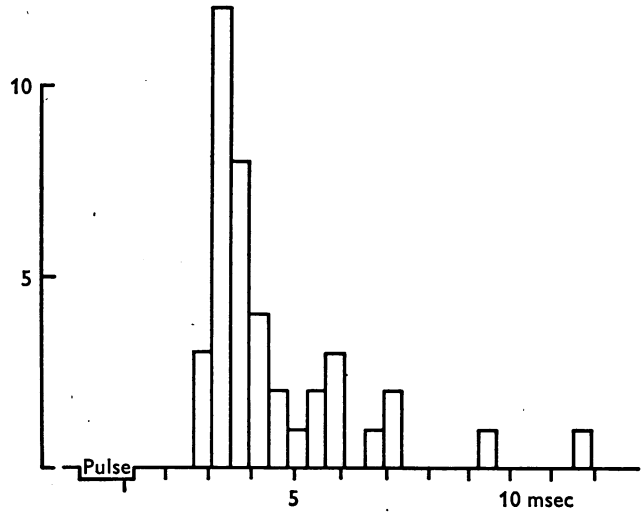

Fig. 3. From same experiment as Figs. 1 and 2. Latency histogram of resolvable unit responses evoked by focal pulses of constant strength. Abscissa: latency of e.p.p. measured from beginning of pulse (as shown). Ordinate: number of observed unit e.p.p.s.

Figure 4 shows a strength–response curve from another experiment in which the 'electrotonic' method was used, i.e. the depolarizing pulses affected the whole of the presynaptic arborization. Other examples obtained by the same technique are shown in Figs. 7*A* and 9*A*.

The conversion of graded into 'triggered' e.p.p.s by TEA

Observations in the presence of Na. The sigmoid relation between applied current and resulting e.p.p. resembles that previously found in TTX-treated preparations (Katz & Miledi, 1967*b*). This kind of strength–response curve was to be expected in the absence of regenerative electric activity, that is under conditions of TTX paralysis or of total replacement of Na by ions like Ca which do not support an action potential.

Now, it has been shown (Katz & Miledi, 1967*c*) that addition of 5 mM-TEA can convert the graded e.p.p. response into one that is virtually of

all-or-none type, so that a very small increment of current intensity gives rise to a very large e.p.p., 40 or 50 mV in amplitude. Further examples of these huge e.p.p.s are shown in Fig. 5. The potential reaches a flat top at a level of approximately -19 mV, which cannot be far from the 'equilibrium potential' (cf. del Castillo & Katz, 1954). This large response is all the more remarkable as a TEA concentration of 5 mM has a strongly curarizing effect and reduces the size of individual m.e.p.p.s to a small

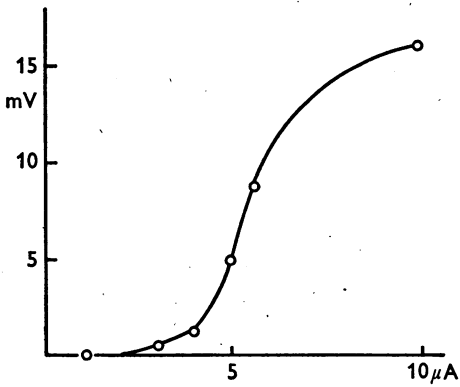

Fig. 4. Strength–response curve in isotonic Ca-Ringer. 5° C 'Electrotonic' method. Abscissa: intensity of 10 msec pulse. Ordinate: size of e.p.p.

fraction (to less than 1/6 and at times to as little as 1/50). The transmitter release associated with these e.p.p.s is clearly much more intense and involves a much larger number of quantal units than that following a normal action potential.

It appears that the nerve terminals not only possess a TTX-resistant mechanism of releasing transmitter in response to local depolarization, but that they are capable of giving a regenerative response under the influence of TEA, in spite of the presence of TTX. Direct evidence for such a self-reinforcing potential change, confined to the presynaptic terminals, has recently been obtained in the giant synapse of the squid (Katz & Miledi, 1969), and it seems very likely that we are dealing here with a similar phenomenon. In the squid experiments there were several indications that influx of Ca during depolarization of the nerve ending can produce a regenerative effect when the counter-current of K has been reduced by TEA. The evidence, however, was incomplete, and the experiment of totally replacing external Na by Ca ions failed to give clear-cut results. It is, therefore, of interest to see now whether the triggered type of e.p.p. can still be obtained in isotonic Ca-Ringer.

Ca-Ringer and TEA. Figures 6 and 8 show examples of e.p.p.s evoked by depolarizing pulses of varying intensities ('electrotonic' method) in a

preparation which was first immersed in isotonic Ca-Ringer to which $2\frac{1}{2}$ hr later 5 mM-TEA was added. The relation between applied current strength and e.p.p. amplitude is shown in Fig. 7, before (*A*) and after (*B*, later changing to *C*) addition of TEA. In TEA, though there is still some gradation, the e.p.p. increases from 0 to 45 mV over an extremely narrow range

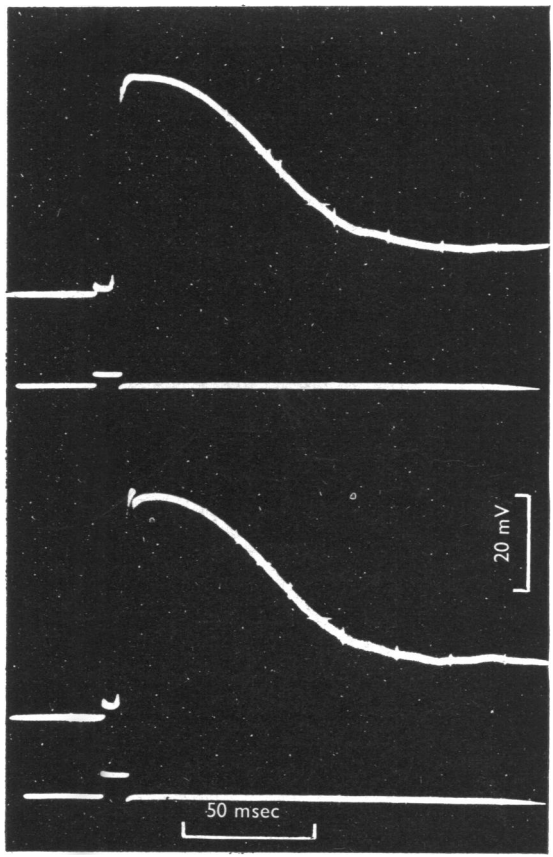

Fig. 5. Triggered 'giant e.p.p.s' obtained in Ringer + 5 mM TEA (+TTX). 9° C. The experiment started with a solution containing zero Ca and 13 mM-Mg, in which no e.p.p.s could be elicited. The recorded responses were obtained after adding 10 mM-Ca. The 'electrotonic' method was used in this and all following illustrations. Voltage scale also represents 4·6 μA, for pulses in lower traces.

of current intensities, which is quite different from the strength/response relation observed without TEA. Further examples are shown in Figs. 8 to 12. To summarize, the effect of TEA in Ca-Ringer is not very different from that in the ordinary ionic environment, though the size and particularly the duration of the giant e.p.p.s were, on the whole, not quite as

large, and during repeated stimulation the response tended to fail more rapidly. Deterioration manifested itself in two ways: the 'threshold' increased as is seen in the lateral shift of curves *B* to *C* in Fig. 7, and the amplitude diminished as illustrated in Figs. 10 and 11.

The transition from no response to maximum e.p.p. varied somewhat in different experiments. For example, gradation was more evident in the case of Fig. 12, where the e.p.p. rose from zero to maximum over a 10%

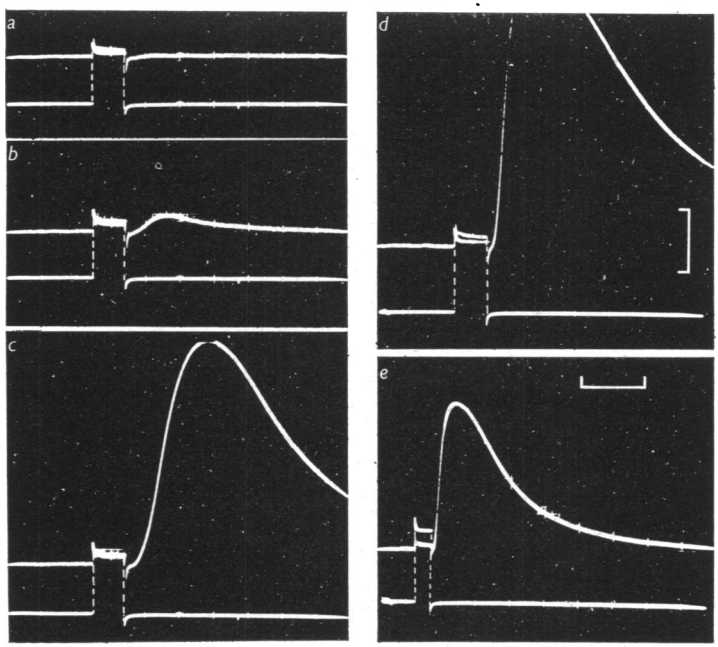

Fig. 6. E.p.p.s in isotonic Ca + 5 mM-TEA. 5° C. *a* to *d*: responses to pulses of increasing strength. Pulses (retouched) monitored in lower trace of each record. *e*: like *d*, but on slower sweep and reduced voltage amplification. Time scale: 20 msec for *a* to *d*, 40 msec for *e*. Volt scale: 10 mV for *a* to *d*, 20 mV for *e*; it represents 4·6 μA for pulses.

increase of stimulus strength, than in Fig. 9 where the e.p.p. was practically all-or-none. Gradation was more pronounced when brief (1–2 msec) rather than long (10 msec) current pulses were used. This is to be expected with local regenerative activity of the kind recorded in the squid giant synapse (Katz & Miledi, 1969); moreover, gradation of the e.p.p. will vary with the spatial decrement of depolarization along the nerve endings and, therefore, depend on the length of the terminals as well as the duration of the pulse. It is of interest that large triggered e.p.p.s could be elicited only by the 'electrotonic' method of stimulation which depolarizes the whole arborization, and not by focal pulse application (with pipettes of up to 7 μ tip

size) which restricts the depolarization to an area which is evidently too small for the local current to become regenerative.

A few other points in Figs. 8 to 12 are worth commenting upon. Figures 10 and 11 show that, with different current intensities, there is a large change in the latency of the e.p.p., but little change in its shape or size, a further indication of the triggered character of the response. Figure 12 shows that hyperpolarizing pulses (which can cause 'break-down' effect

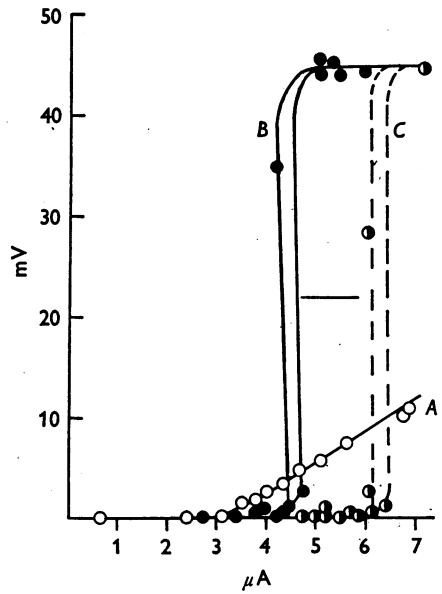

Fig. 7. From same experiment as Fig. 6. Strength–response curves in isotonic Ca-Ringer. *A* (open circles): before adding TEA. *B* (filled circles) and *C* (half-filled circles): after adding 5 mM-TEA. Abscissa: intensity of 10 msec pulses. Ordinate: size of e.p.p.

and high-frequency outbursts of m.e.p.p.s) did not trigger the e.p.p. at current intensities more than twice the depolarizing 'threshold'. Figures 8 and 9 show that even after addition of a large dose of TTX to the Ca + TEA-Ringer, the e.p.p. response could still be obtained.

Finally, when current pulses were applied directly to the muscle fibre via a second intracellular electrode, no sign of a regenerative potential was seen. The voltage–current relation, beyond a linear range of 60–80 mV depolarization, showed 'delayed rectification', associated usually with local contraction.

It may be mentioned that no sign of an action potential could be recorded from the desheathed sartorius nerve, after treatment with isotonic Ca + TEA. This is not unexpected, for in related experiments on the squid (Katz & Miledi, 1969) the regenerative response was found to be confined to the presynaptic terminals.

Amplitude and reversal point of e.p.p. in Ca+TEA-Ringer. Regarding the size of the e.p.p., values of up to 45 mV (starting from a resting potential of −95 mV) were recorded. Considering that in the Ca+TEA-Ringer, individual m.e.p.p.s were no longer detectable (their amplitudes must have

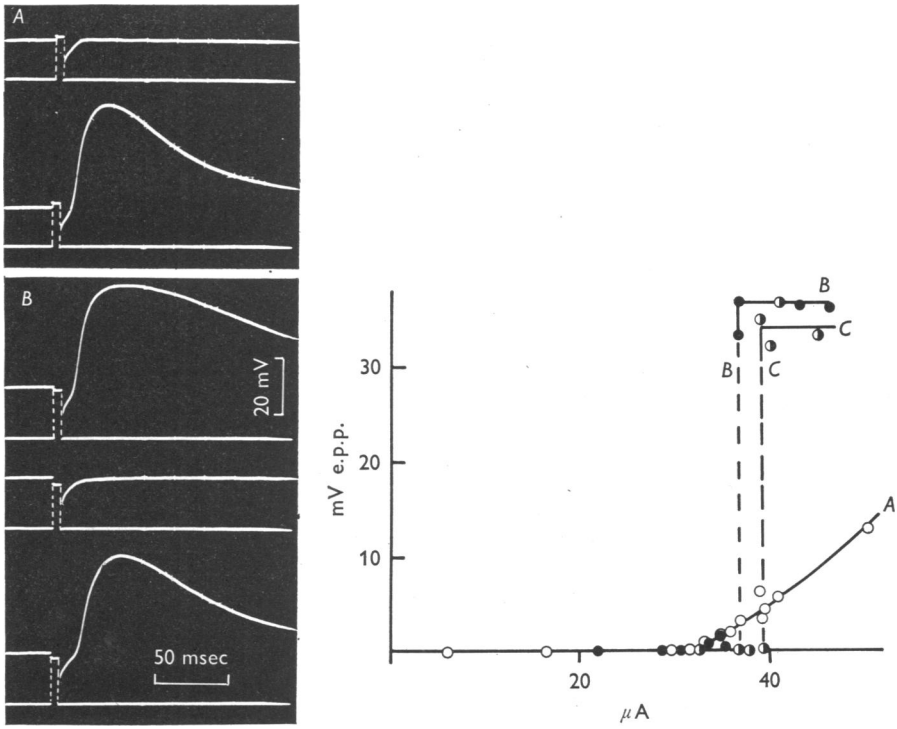

Fig. 8. E.p.p.s in Ca-Ringer+TEA, with addition of TTX. 6° C. In *A*, TTX concentration was 10^{-6} g/ml.; in *B*, $1{\cdot}1 \times 10^{-5}$ g/ml. Subthreshold and superthreshold pulses are shown (monitored in lower trace of each record). The first (long) response in *B* was obtained after a longer period of rest than the others. Volt scale represents also 47·5 μA. (Note: current intensity was high in this experiment, because a thick branch of the nerve was used.)

Fig. 9. From same experiment as Fig. 8. Strength–response curves in Ca-Ringer. *A*: before adding TEA. *B* and *C*: after adding 5 mM-TEA. TTX concentration: 10^{-6} g/ml. in *A* and *B*; $1{\cdot}1 \times 10^{-5}$ g/ml. in *C*. Abscissa: strength of 5 msec pulses. Ordinate: size of e.p.p.

been less than 50–100 μV), the triggered e.p.p. represents an enormous release, of many thousands of 'packets', similar to that in Na+TEA-Ringer (cf. Katz & Miledi, 1967*c*; see also Discussion).

In several experiments, attempts were made to determine the 'reversal potential' of the e.p.p. in Ca+TEA-Ringer. In the experiment illustrated

in Fig. 13, the observed 'null-point' was at approximately -54 mV, but as the recording electrode was about 0·25 mm from the centre of the junction, the true reversal potential may have been several millivolts more positive. At two other junctions of the same muscle, reversal levels of approx. -35 mV and -39 mV were found.

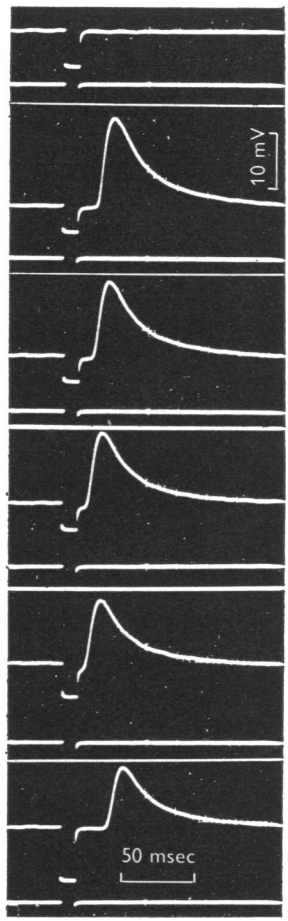

Fig. 10. E.p.p.s in Ca-Ringer + TEA, showing change of latency with different current intensities. 4·5° C. Pulses (monitored in lower trace of each record) were applied in the order shown, from above downwards. There was progressive reduction in e.p.p. amplitude (cf. Fig. 11). Volt scale also represents 9·25 μA.

Long-term effects and 'after-effects' of isotonic Ca-Ringer

With the preparation kept at low temperature (about 6–7° C), e.p.p. responses could be obtained in some experiments even after 5–6 hr immersion in isotonic Ca-Ringer, the responses being of the triggered or graded

variety depending on whether TEA had been added or not. The triggered e.p.p.s could not be repeated indefinitely, but tended to deteriorate and ultimately vanish after a comparatively short series of stimuli. It is difficult to be precise about the number, because numerous test stimuli were given while searching for a muscle fibre which was supplied by the fine nerve twig (see Methods), and each pulse presumably evoked local responses in all the connected junctions.

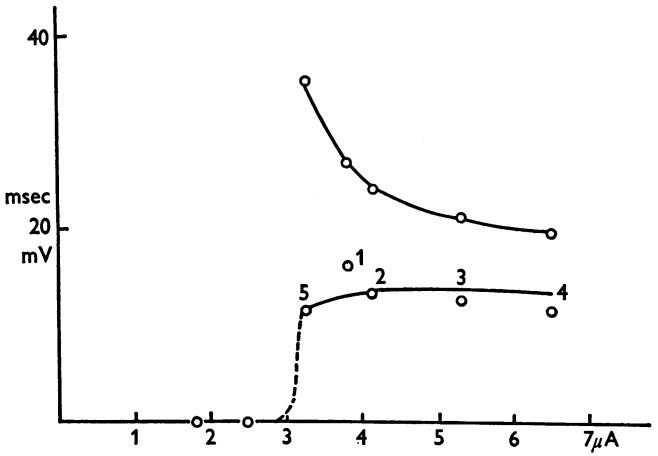

Fig. 11. Same experiment as Fig. 10. Lower curve: strength–response curve. The numbers on each point show the sequence in which pulses were applied. Upper curve: latency of e.p.p., measured from start of pulse (approx. 10 msec long) to half-rise of e.p.p. Abscissa: strength of current pulse. Ordinates: lower curve: e.p.p. size in mV; upper curve: latency in msec.

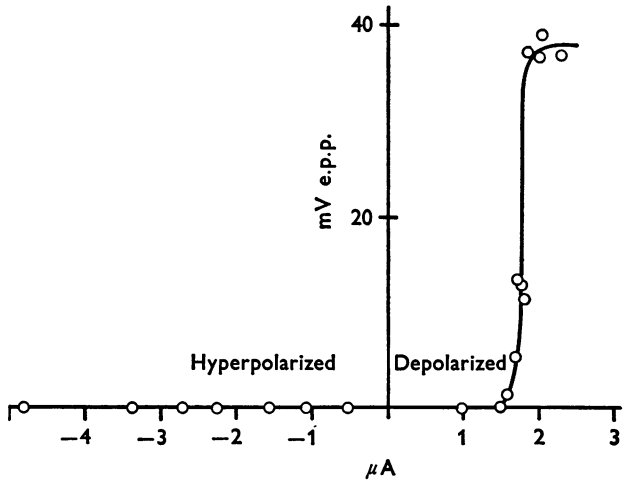

Fig. 12. Strength–response curve in Ca-Ringer + TEA, covering a range of hyperpolarizing intensities.

But even in the absence of TEA and when no stimuli were given, the properties of the nerve endings underwent irreversible changes after several hours exposure to the isotonic Ca bath. The general picture, at 7° C, was as follows (although the time scale varied a good deal in different experiments): within 1–3 hr after exposure to isotonic Ca, the frequency of m.e.p.p.s rose and in some fibres reached very high values. During the next few hours, the spontaneous discharge rate fell and eventually, after periods of exposure varying between 4 and more than 7½ hr, the frequency was much lower

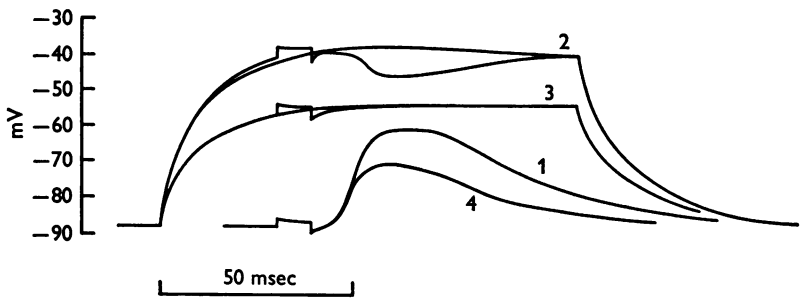

Fig. 13. Tracings showing 'reversal' of e.p.p. in isotonic Ca + TEA. E.p.p.s were elicited at three different levels of post-synaptic membrane potential, which was displaced by current applied to the muscle fibre through a second intracellular electrode. The numbers on the curves show the sequence in which pulses were applied to the nerve.

than initially, and in many fibres spontaneous activity seemed to have stopped. During this period e.p.p.s could no longer be evoked by locally applied depolarization, and this failure appeared to be irreversible.

If at this stage the bath was changed to the normal ionic environment (with or without TTX), there was an immediate resumption of spontaneous (not of evoked) end-plate activity. This was characterized by a most unusual amplitude distribution, altogether different from that observed normally or *during* the period of Ca exposure. It is illustrated in Fig. 14 which also shows, for comparison, spontaneous m.e.p.p.s during Ca treatment, at a time when the frequency was high. The spontaneous potentials, after delayed return to Ringer, were generally of low frequency and varied in amplitude over an enormous range. For example, in the fibres shown in Fig. 14*B* to *D*, the amplitudes of the spontaneous e.p.p.s varied between less than 0·1 and over 35 mV. It was indeed necessary to add TTX before readmitting the Ringer solution in order to avoid spontaneous twitching.

It seems that the prolonged exposure to the isotonic Ca-Ringer had somehow disorganized the motor nerve endings and produced an abnormal mode of spontaneous 'secretion'.

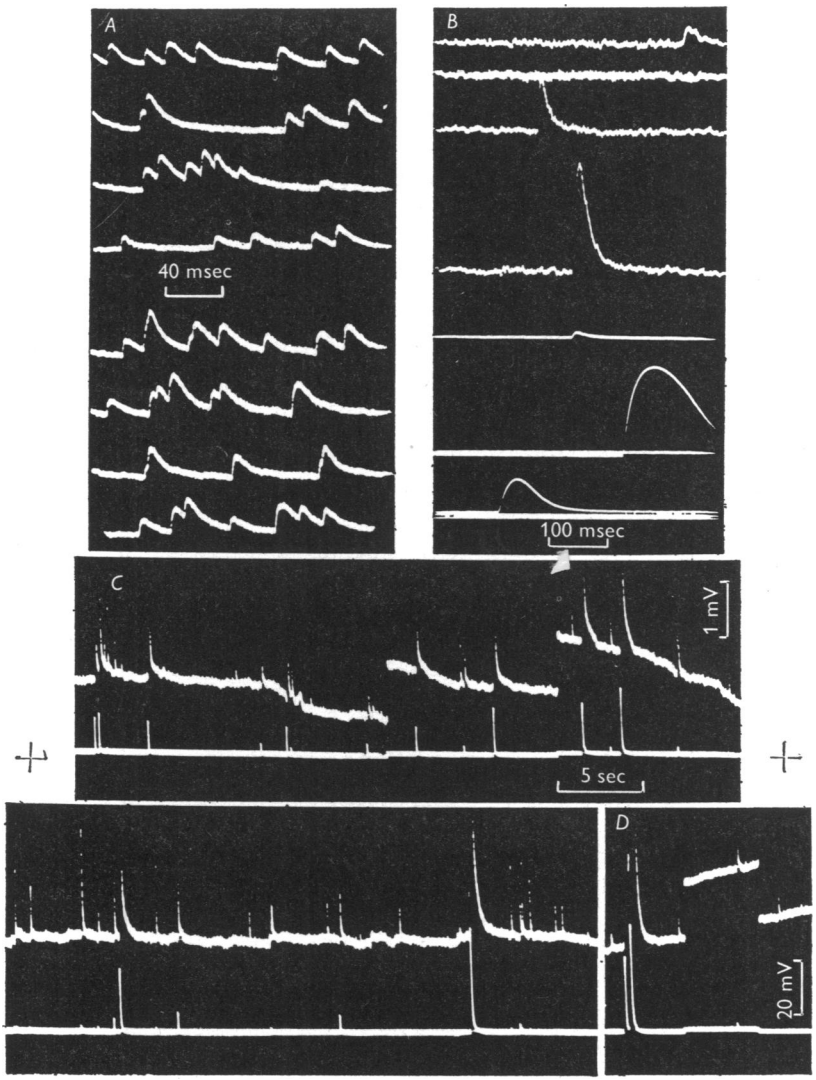

Fig. 14. Spontaneous end-plate activity. No TEA. *A*: **in isotonic Ca, after 2 hr 45 min. The temperature of the bath had risen to 20° C.** *B* **to** *D*: **after delayed return, from isotonic Ca, to Na-Ringer (+TTX). This preparation had been kept for 6 hr at 11° C in Ca-Ringer; at the end of this period the frequency of m.e.p.p.s had fallen to a very low value. In** *B*, **upper four traces at high gain (1 mV scale), lower traces at low gain (20 mV scale); 4th and 5th trace show the same signal (recorded simultaneously). In** *C* **and** *D*, **high gain (upper) and low-gain (lower) recordings were made simultaneously to cover the very wide range of amplitudes.**

DISCUSSION

A. *Transmitter release and post-synaptic action in isotonic Ca*

It had previously been shown that the abolition of Na influx by TTX does not stop the release of ACh from nerve endings, in response to local depolarization. The essential immediate requirements for transmitter release appeared to be Ca rather than Na in the external medium. The present experiments provide further evidence in showing that e.p.p.s can be evoked during complete absence of external Na and its isotonic replacement by Ca.

The experiments also confirm the observations of Takeuchi (1963), viz. that in Ca-Ringer, ACh remains an effective depolarizing agent, though its potency is greatly diminished. M.e.p.p.s are reduced to about 1/6 in amplitude by isotonic Ca-substitution. It is interesting that m.e.p.p.s of similar size are obtained in isotonic Mg-Ringer. It appears that the ACh-operated ionic 'gates' in the post-synaptic membrane do not discriminate very much between Ca and Mg ions. This contrasts with the presynaptic release sites where Mg acts as a competitive blocking agent.

It remains uncertain whether this competition occurs on the outer membrane surface, that is whether Mg interferes with Ca entry, or whether Mg can traverse the membrane and blocks the reactive sites on the inner surface.

While the inward end-plate current in the present experiments (with 0 mM $[Na_o]$ and 83 mM-$[Ca_o]$ is presumably made up chiefly or entirely by influx of Ca ions, the contribution of Ca current to the e.p.p. in a normal environment (1·8 mM-$[Ca_o]$ and 115 mM-$[Na_o]$) must be very much smaller and is probably negligible.

B. *The action of TEA*

The effect of TEA at the neuromuscular junction is twofold: it acts like curare on post-synaptic receptor sites (cf. Nastuk, 1959; Koketsu, 1958), and yet it has long been known to have a 'decurarizing' action (Kensler, 1950; Koketsu, 1958). This is due to an enormous potentiation of transmitter release, whether by a nerve action potential or by a local depolarizing pulse in TTX or isotonic Ca-treated preparations.

The potentiation in a normal or curarized preparation is fairly easy to explain, for TEA is known to lengthen the duration of the action potential (Hagiwara & Tasaki, 1957; Koketsu, 1958; Schmidt & Stämpfli, 1966), and this is associated with a large increase in transmitter release from the endings (cf. Katz & Miledi, 1967*a*; Mambrini & Benoit, 1968). But the potentiation by TEA in the absence of nerve impulses, after TTX or isotonic Ca treatment, is a more curious phenomenon; it suggests that TEA somehow enables the nerve endings to produce an electric response, even

when the rest of the system is unable to generate an action potential. Direct evidence for such a presynaptic response has recently been obtained in the stellate ganglion of the squid (Katz & Miledi, 1969). The present experiments lend support to the view that such local activity can occur in the absence of external Na and that Ca inward current in nerve endings can become regenerative.

There are several possible ways in which TEA could bring about the trigger effect. Beaulieu & Frank (1967*a*, *b*) have produced evidence which indicates that TEA facilitates movement of Ca through the cell membrane; this could be the basis of the regenerative action. It should be noted, however, that the well known effect of TEA in blocking K efflux (see Armstrong & Binstock, 1965) may itself be sufficient to enable a small inward current (of Ca or other cations) to become regenerative, without having to invoke specific effects of TEA on Ca permeability. The relative importance of these two factors still remains to be clarified.

C. *The delayed effects of isotonic Ca*

Further problems are presented by the progressive deterioration of the triggered response, and by the ultimate irreversible changes of the nerve endings which occur in the Ca-Ringer. An obvious possibility is that both these phenomena result from gradual accumulation of Ca inside the terminals, caused by the high external Ca concentration and perhaps aggravated by the lack of external Na which, apart from competing for Ca entry, appears to facilitate extrusion of Ca from the axoplasm (Blaustein & Hodgkin, 1969).

Certain interesting possibilities arise in connexion with the gradual cessation of m.e.p.p.s, and their highly abnormal size distribution after return to Na-Ringer. Accumulation of Ca inside the terminals might be expected to produce structural changes, and it is clearly of interest to follow the different stages of the Ca effect by parallel observations with the electron microscope. It is hoped to report the results of such a study in a later paper.

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