

EFFECT OF
PROSTIGMINE ON THE TIME COURSE OF THE END-PLATE
POTENTIAL IN THE RAT DIAPHRAGM

BY K. KUBA AND T. TOMITA

*From the Department of Physiology, Kyushu University,
Fukuoka, Japan*

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SUMMARY

1. End-plate potentials (e.p.p.s) were evoked by applying brief depolarizing pulses to motor nerve endings in a phrenic nerve–diaphragm preparation paralysed by tetrodotoxin (TTX). Without prostigmine, the time to decay from the summit of the e.p.p. to half amplitude (the half-decay time) was roughly constant (2–4 msec) when the amplitude was increased by increasing stimulus intensity or duration.

2. In the presence of prostigmine (5×10^{-7} – 2×10^{-5} g/ml.), the falling phase of the e.p.p. was different in time course depending on the amplitude. The half-decay time had, very roughly, an exponential dependence on amplitude. The relationship was not affected by increasing the TTX or the prostigmine concentration, but D-tubocurarine (10^{-6} g/ml.) made the relationship less steep.

3. Hyperpolarizing current pulses, applied after the depolarizing current pulse which produced the e.p.p., had no effect on the time course of the e.p.p. No facilitating effect of repetitive stimulation was observed without prostigmine up to a frequency of 40 c/s, but there was a strong effect of repetitive stimulation in increasing the amplitude and duration of the e.p.p. in the presence of prostigmine. During stimulation, the end-plate was continuously depolarized by 10–20 mV, and its recovery was very slow, the half-decay time being about 3 sec.

4. The half-decay time of the acetylcholine potentials produced by iontophoretically applied acetylcholine was almost independent of the amplitude, with or without prostigmine, although it increased the amplitude of the potential and prolonged the falling phase.

5. Possible mechanisms for the alteration of the falling phase of the e.p.p. were discussed. It is speculated that, in the presence of prostigmine, a process which is involved in a conductance increase of the post-synaptic membrane, after acetylcholine has combined with the receptor molecules, is the main factor determining the falling phase of the e.p.p.

INTRODUCTION

It is known that in normal solution the main part of transmitter action at the neuromuscular junction ceases within the rising phase of the end-plate potential (e.p.p.), and that the falling phase of the e.p.p. is to a large extent determined by the electrical time constant of the muscle membrane (Fatt & Katz, 1951). However, in the presence of a cholinesterase inhibitor such as a prostigmine, the decay of the e.p.p. is prolonged. This is assumed to be due to the survival of acetylcholine in the junctional region on account of cholinesterase inactivation (Eccles & MacFarlane, 1949). In this condition, the disappearance of transmitter action has mainly been explained by diffusion of transmitter out of the junctional cleft (Eccles & Jaeger, 1958).

During experiments, in which the effects of catecholamine on the neuromuscular junction were investigated in the rat diaphragm (Kuba, 1971), it was noted that the falling phase of small e.p.p.s evoked by applying depolarizing pulses to nerve terminals was much faster than that of large e.p.p.s when the preparation was treated with prostigmine. This observation prompted us to investigate the time course of the e.p.p. in the presence of prostigmine in some detail.

There are several possible explanations for the difference in time course of the e.p.p.s. At an early stage of the experiments, it was thought that a diffusion process was the main factor causing the difference in the falling phase of the e.p.p.s. However, some other processes, involved in the conductance increase of the post-synaptic membrane after acetylcholine has combined with the receptor, are probably more important in determining the time course of the e.p.p. in the presence of prostigmine.

METHODS

Rats weighing 100–150 g were stunned and bled under ether anaesthesia. The phrenic nerve–hemidiaphragm preparation was dissected and the muscle was cut out along the direction of the fibres to a 1.5–2.0 cm wide strip. It was mounted in a lucite chamber with a capacity of about 5 ml. The muscle was bathed in oxygenated Krebs solution containing tetrodotoxin (10^{-7} – 10^{-6} g/ml.) which suppressed action potentials in nerve and muscle fibres. In order to evoke the e.p.p. a depolarizing current pulse was applied to the nerve terminal as described by Katz & Miledi (1965, 1967). A glass micropipette, usually of 10–30 μ tip diameter, filled with 1.5–2.0 M-NaCl solution, was placed on the nerve terminal. Current pulses were passed between this electrode and the indifferent Ag–AgCl electrode in the bath.

Intracellular recording was done with glass micropipettes filled with 3 M-KCl, of resistances ranging from 10 to 30 M Ω . The localization of the nerve terminal was made as follows. At first, an intracellular electrode was inserted into the end-plate region so as to record the miniature end-plate potentials (m.e.p.p.s) of large amplitude. Then, a stimulating electrode was brought on the muscle fibre near the record-

ing electrode with the aid of a micro-manipulator, passing depolarizing pulses through the electrode at a constant rate, until a large post-junctional response was obtained.

Post-synaptic potentials were also produced by iontophoretic application of acetylcholine. The current pulses (10^{-8} – 10^{-7} A in intensity and 0.1–10 msec in duration) were applied to an electrode containing acetylcholine (1.5–2 M). Spontaneous diffusion of acetylcholine from the electrode was prevented by applying a negative potential to the electrode as described by Krnjević & Miledi (1958).

The bathing solution was a modified Krebs solution containing (mM) Na 137, K 5.9, Mg 1.2, Ca 2.5, Cl 134, H_2PO_4 1.2, HCO_3 15.5, glucose 11.5. It was equilibrated with a mixture of 97% O_2 and 3% CO_2 . The temperature was kept constant at about 30° C. The drugs used were prostigmine (Shionogi), D-tubocurarine (Sigma), and tetrodotoxin (Sankyo).

Effects of prostigmine were observed in the same junction usually 10–30 min after prostigmine application, because of difficulty in keeping the micro-electrodes in place longer than 30 min. The exposure for less than 30 min to prostigmine, 5×10^{-7} g/ml., was probably not sufficient for complete cholinesterase inhibition. However, the main points in the results did not seem to be affected.

RESULTS

As found previously by Katz & Miledi (1965, 1967), end-plate potentials (e.p.p.s) can be produced by applying current pulses of graded strength and duration to the motor nerve endings in which the nerve impulses have been eliminated by the presence of tetrodotoxin (TTX 10^{-7} g/ml.). Examples of such potentials are shown in Fig. 1, which were elicited by current pulses of various intensities but constant duration of 0.2 msec. At the beginning of the e.p.p.s there were often electrical artifacts produced by current pulses. The artifact was larger with higher resistance of micro-electrodes used. However, it did not influence the falling phase of the e.p.p.s.

The time to decay from the summit to half amplitude (the half-decay time) of the e.p.p. was only slightly prolonged when the e.p.p. amplitude was successively increased by raising the current intensity, as shown in Fig. 1, or by increasing the duration (0.1–1.0 msec) of the current pulse. An increase in amplitude of the e.p.p. became progressively less beyond about 10 mV as the stimulus intensity or duration was increased step-wise. However, even in such a large response the half-decay time remained more or less the same (about 2.5 msec).

Effect of prostigmine. When prostigmine was applied at concentrations of 5×10^{-7} – 2×10^{-5} g/ml., the falling phase of the e.p.p. varied in time course depending on the amplitude of the e.p.p. Fig. 2 shows the e.p.p.s evoked by passive depolarization of the nerve terminal (A, B) and miniature end-plate potentials (m.e.p.p.s) (C) in a solution containing TTX (10^{-7} g/ml.) and prostigmine (5×10^{-7} g/ml.). All responses were obtained

from the same end-plate region. The falling phase of the e.p.p. was slowed and prolonged as the amplitude of the e.p.p. was increased.

In Fig. 3, the relationships between the peak amplitude and the half decay time of the e.p.p.s and of the m.e.p.p.s are plotted. In the presence of prostigmine, the half-decay time of the e.p.p. increased very roughly

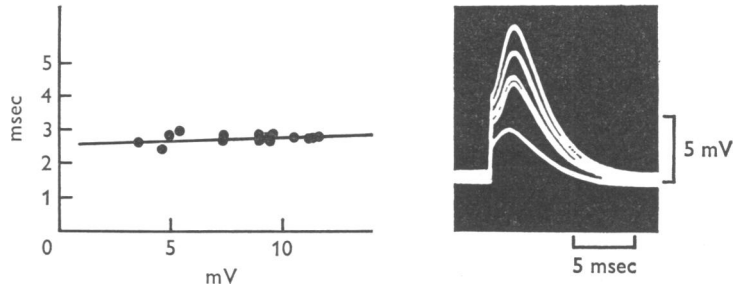


Fig. 1. Picture: examples of e.p.p.s of rat diaphragm, evoked by 0.2 msec pulses of various intensities ($4\text{--}20\ \mu\text{A}$) applied to a nerve terminal in the presence of TTX ($10^{-7}\ \text{g/ml.}$). Pulses applied at intervals of 10 sec. Graph: relationship between amplitude (abscissa) and half-decay time (ordinate) obtained from the experiment shown in the picture.

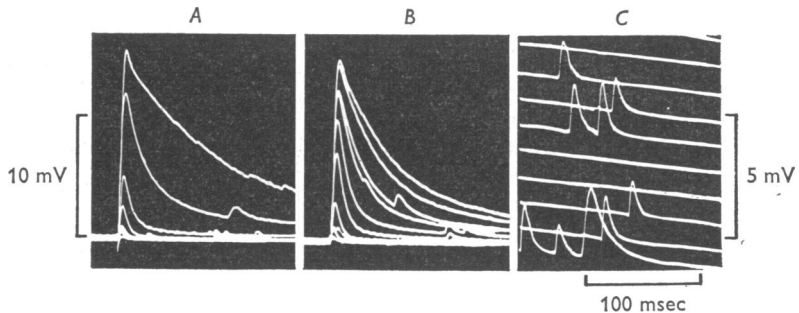


Fig. 2. E.p.p.s (A, B) evoked by brief depolarizing pulses applied to a nerve terminal, and m.e.p.p.s (C) in the presence of prostigmine ($5 \times 10^{-7}\ \text{g/ml.}$) and tetrodotoxin ($10^{-7}\ \text{g/ml.}$). A: intensity of current pulses was changed ($2\text{--}16 \times 10^{-6}\ \text{A}$) at constant duration (0.6 msec). B: duration was changed (0.1–1.0 msec) at constant intensity ($14 \times 10^{-6}\ \text{A}$).

exponentially as the amplitude increased. As seen in the Figure, the amplitude–half-decay time relationship of the m.e.p.p. (filled circles) fell within the same curve of the e.p.p.

The amplitude–half-decay time relationship differed from one preparation to another, but it was rather constant in any single preparation and not influenced by increasing TTX concentration from 10^{-7} to $10^{-6}\ \text{g/ml.}$, or by increasing prostigmine from 10^{-6} to $2.5 \times 10^{-5}\ \text{g/ml.}$ (Fig. 4).

The application of D-tubocurarine at a concentration of $10^{-6}\ \text{g/ml.}$

shortened the falling phase of the e.p.p. within 5–10 min (Fig. 5*b*), which had been prolonged by prostigmine (10^{-6} g/ml.) (Fig. 5*a*). Therefore, the amplitude–half-decay time relationship became less steep as shown in

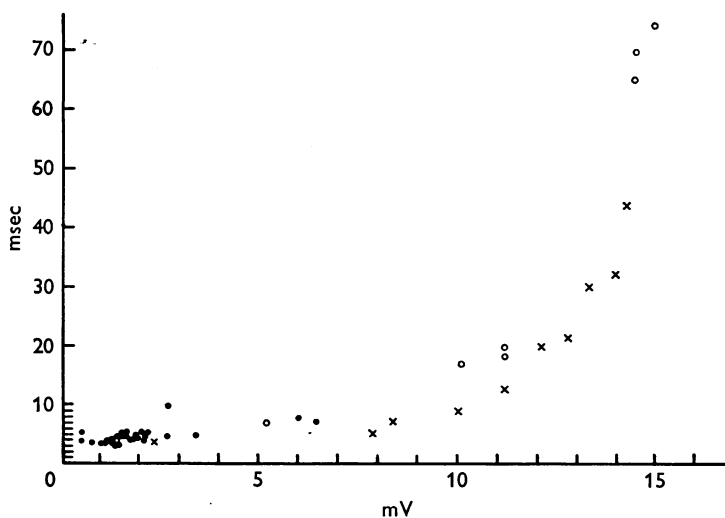


Fig. 3. Relationship between amplitude and half-decay time of e.p.p.s and of m.e.p.p.s obtained from the experiment shown in Fig. 2. Abscissa: amplitude; ordinate: half-decay time of e.p.p. or m.e.p.p. Open circles: e.p.p.s evoked by varying intensity; crosses: e.p.p.s evoked by varying duration; filled circles: m.e.p.p.s.

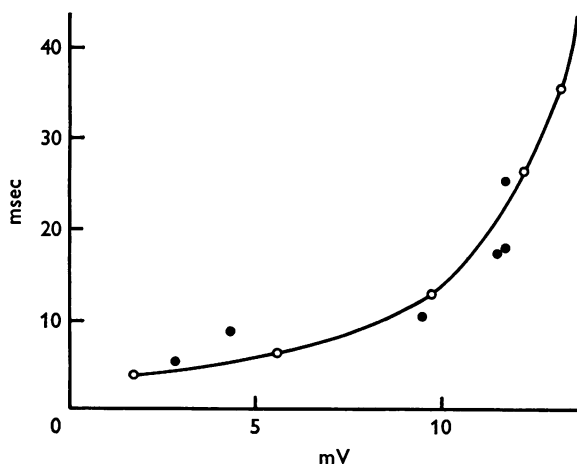


Fig. 4. Effect of increase in concentrations of prostigmine on relationship between amplitude of e.p.p. (abscissa) and half-decay time (ordinate). Prostigmine: open circles, 1×10^{-6} g/ml.; filled circles, 2.5×10^{-5} g/ml. TTX: 10^{-7} g/ml. Current pulses applied to nerve terminal: 1 msec duration, $4-29 \times 10^{-6}$ A. More than 20 min exposure to prostigmine.

Fig. 5. In the frog sartorius muscle, a similar shortening of the e.p.p. prolonged by prostigmine has already been observed by application of tubocurarine (Beránek & Vyskočil, 1968; Kordaš, 1968).

Effect of second hyperpolarizing current pulse. It may be that a prolonged falling phase of the e.p.p. in the presence of prostigmine is due to a long lasting depolarization leading to a more prolonged release of acetylcholine

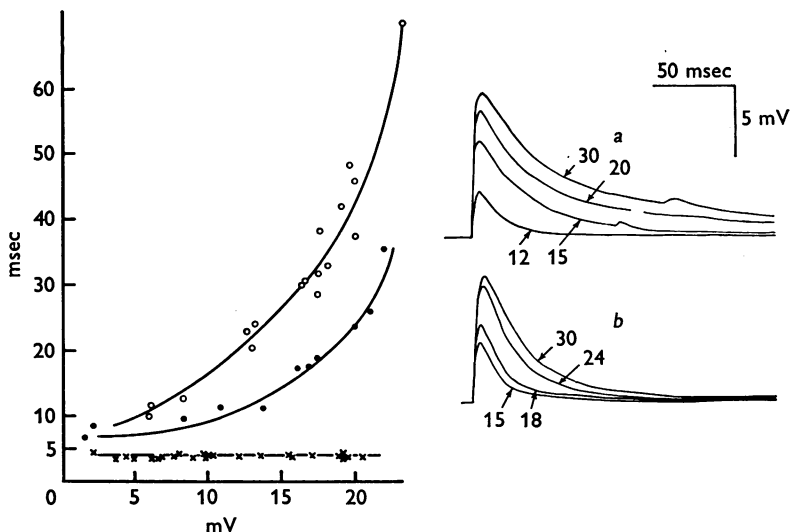


Fig. 5. Effect of prostigmine (10^{-6} g/ml., open circles) and addition of D-tubocurarine (10^{-6} g/ml., filled circles) on relationship between amplitude of e.p.p.s and half-decay time. Crosses: control in normal solution. All contain TTX (10^{-7} g/ml.). Current pulses applied to nerve terminal: 0.2 msec, $3-30 \times 10^{-6}$ A. Tracings show examples of e.p.p.s before (a) and 10 min after treatment with D-tubocurarine (b). Numbers indicate current intensity ($\times 10^{-6}$ A) applied to nerve terminal.

from the nerve terminal. To test this possibility, a hyperpolarizing current pulse was applied to the nerve terminal within a short interval (1–5 msec) after the first depolarizing current pulse. An example of such experiments is shown in Fig. 6b. There was no effect on the amplitude or on the time course. Even when the second pulse was applied at various intervals after the first pulse, the time course of the e.p.p. evoked by the first pulse was unchanged. The intensity of the second current pulse was enough to evoke an e.p.p. when applied with reversed polarity (c). When both pulses were applied successively in depolarizing direction, a large and prolonged e.p.p. was observed (d).

Effect of repetitive stimulations. Fig. 7 shows the e.p.p.s evoked by repetitive stimulations of the nerve terminal with current pulses of

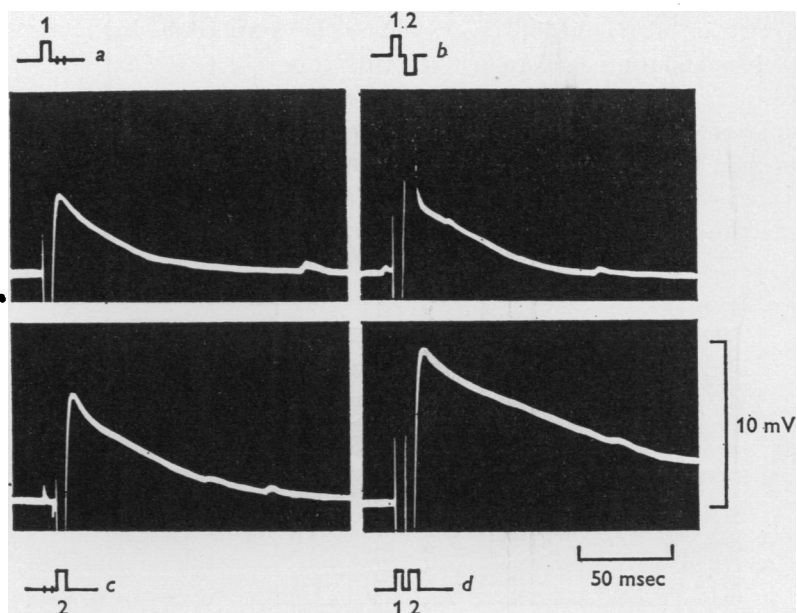


Fig. 6. Effect of hyperpolarizing pulses on e.p.p.s. First pulse: depolarizing current of 3 msec, 12×10^{-6} A. Second pulse: 4 msec, 12×10^{-6} A, applied 2 msec after the first pulse. *a*: first pulse only; *b*: first and second hyperpolarizing pulse; *c*: second depolarizing pulse only; *d*: first and second depolarizing pulse. TTX, 10^{-7} g/ml., prostigmine, 10^{-6} g/ml.

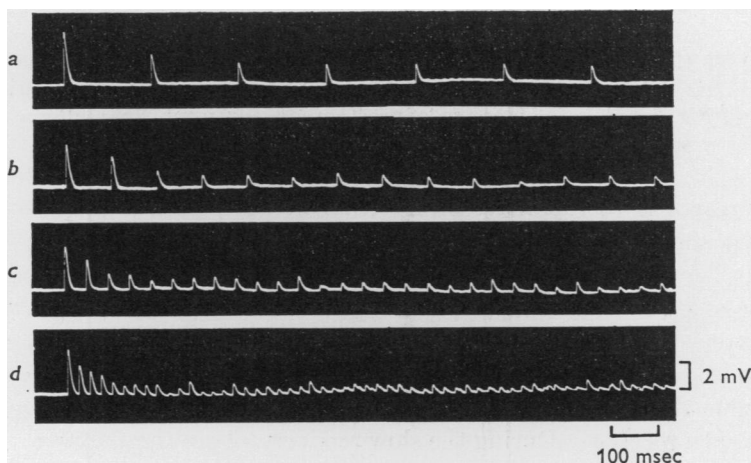


Fig. 7. E.p.p.s in response to repetitive stimulation in normal solution containing TTX (10^{-7} g/ml.). *a*: 5; *b*: 10; *c*: 20; *d*: 40 c/s. Current pulses applied to nerve terminal: 0.2 msec duration, 16×10^{-6} A.

0.2 msec, $16 \mu\text{A}$, at various frequencies (5–40 c/s). In the solution containing TTX (10^{-7} g/ml.) but not prostigmine, the amplitude diminished successively without much change in the time course of each e.p.p.

Prostigmine (10^{-6} g/ml.) drastically modified the responses to repetitive stimulations as shown in Fig. 8. The e.p.p. was very much potentiated and prolonged. There was a sustained depolarization of the membrane which recovered with a very slow time course (a half-decay time of about 3 sec). During this recovery phase, the frequency of the m.e.p.p. was increased.

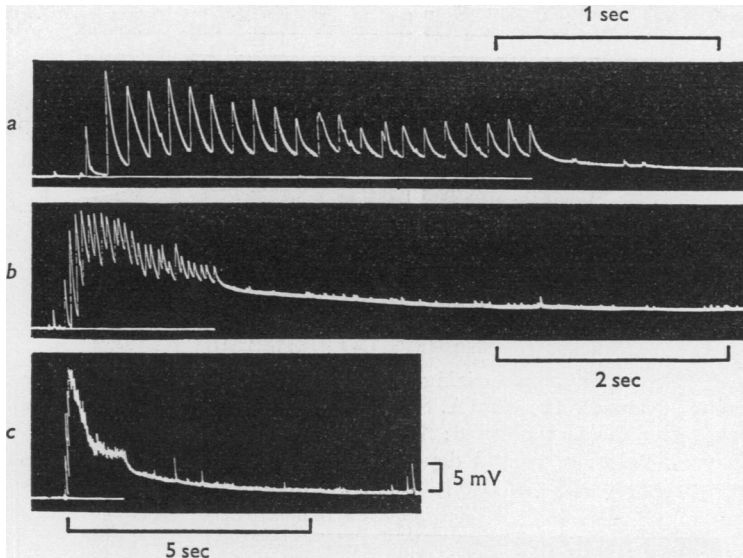


Fig. 8. E.p.p.s evoked by repetitive stimulation applied to the nerve terminal (0.1 msec, 12×10^{-6} A). *a*, 10; *b*, 50; *c*, 200 c/s. Same preparation as in Fig. 7, but in the presence of prostigmine, 10^{-6} g/ml. Note differences in time scale in each record.

The response to repetitive stimulation in the presence of prostigmine was very similar to that observed in frog sartorius by Eccles & MacFarlane (1949). They observed that on cessation of repetitive stimulation the response showed two clearly separable phases of decay with respective half-decay times of 10–30 msec and 0.7–2.5 sec. Although they attributed this slow component to diffusion of acetylcholine from the end-plate region, it is possible that accumulation of potassium ions in the cleft is responsible for the slow recovery. During the slow recovery phase the frequency of the m.e.p.p. was increased. This is probably similar to the observation that repetitive nerve impulses increase m.e.p.p. frequency even when the impulses do not directly release much transmitter (Miledi & Thies, 1967). It could also be due to accumulation of potassium ions in the synaptic cleft.

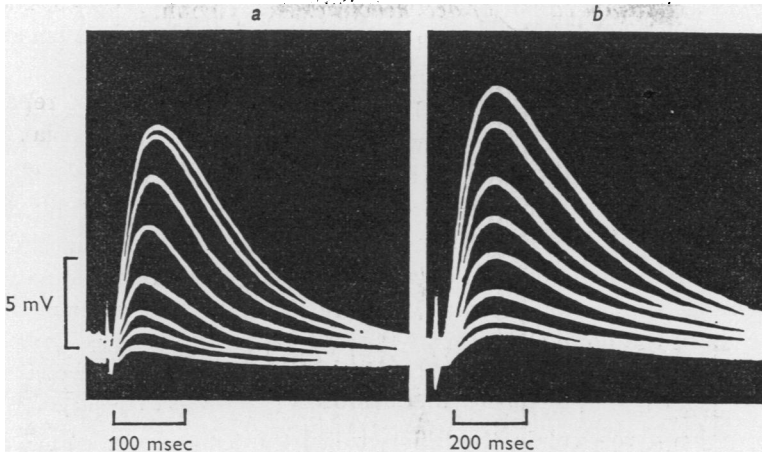


Fig. 9. Responses produced by iontophoretically applied acetylcholine (5 msec current pulses of $4-32 \times 10^{-8}$ A). TTX: 10^{-7} g/ml., with (b) and without (a) prostigmine (5×10^{-7} g/ml., 5 min exposure).

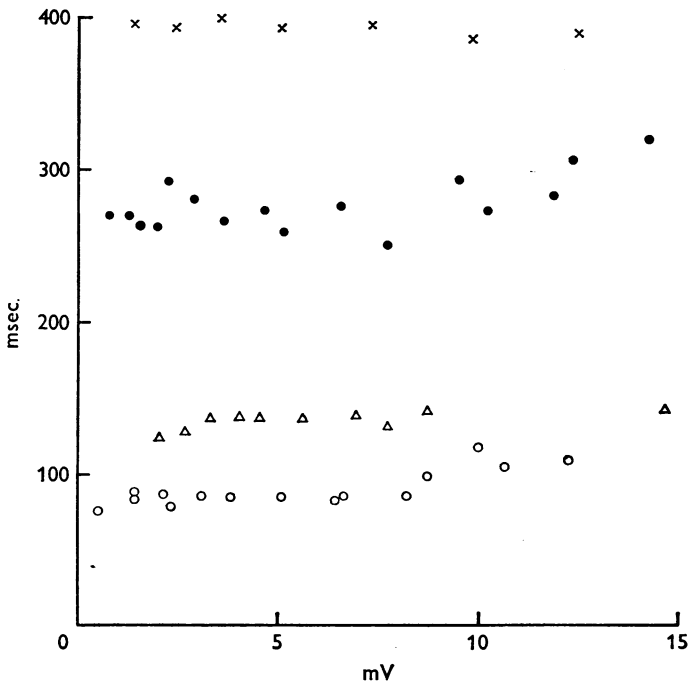


Fig. 10. Relationship between amplitude of acetylcholine potential and half-decay time in the presence of prostigmine (5×10^{-7} g/ml.), obtained from experiments shown in Fig. 9. Open circles: control without prostigmine; triangles: 3 min; filled circles: 5 min; crosses: 10 min exposure to prostigmine. All contain TTX (10^{-7} g/ml.).

Iontophoretic application of acetylcholine. Acetylcholine potentials were produced in the presence of TTX (10^{-7} g/ml.) by iontophoretic application of acetylcholine from a micropipette placed on a sensitive spot of the muscle membrane (Nastuk, 1953; del Castillo & Katz, 1954). Current pulses of $4-32 \times 10^{-8}$ A, 5 msec were applied to the micro-electrode to eject acetylcholine (Fig. 9a). When the preparation was treated with prostigmine (5×10^{-7} g/ml.), both the amplitude and the half-decay time were increased (b). The amplitude-half-decay time relationship is plotted in Fig. 10. Although the half-decay time was gradually prolonged after prostigmine application, it was roughly constant independent of the amplitude of the potential.

DISCUSSION

It is difficult to explain the difference in the time course of the falling phase of the e.p.p. from evidence obtained in the present experiments. However, some factors may be excluded and some possibilities may be suggested.

Since an anticholinesterase, prostigmine, is being used, the elimination of the released transmitter from the junctional cleft is probably determined by diffusion. It may be possible, however, that some esterase activity remains even in the presence of prostigmine at a concentration of 10^{-6} g/ml. The enzyme would then be capable of hydrolysing a small amount of acetylcholine, so that a small e.p.p. has a fast time course. When larger amounts of acetylcholine are released, the portion of acetylcholine destroyed by esterase is relatively small and a decrease in concentration would be determined mainly by diffusion out of the junctional cleft. This is rather unlikely because a further increase in the concentration of prostigmine to 2.5×10^{-5} g/ml. had no further effect on the amplitude-half-decay time relationship of the e.p.p.

A large amount of transmitter might produce saturation of junctional receptors, and this might lead to prolongation of the falling phase of a large e.p.p. Since there is a large increase in amplitude when repetitive stimulation is applied in the presence of prostigmine, as shown in Fig. 8, the receptor area is not saturated even when the prolongation of the e.p.p. is clearly observed.

Another factor involved in slowing the falling phase of the e.p.p. might be a presynaptic factor. Acetylcholine may depolarize the motor nerve terminal, especially in mammalian muscles (Masland & Wigton, 1940; Riker, 1966). It could be that the acetylcholine liberated acts on the terminal so as to increase the release of acetylcholine. If such a mechanism were working, the hyperpolarizing current pulse applied to the terminal shortly after the depolarizing pulse should modify the time course of the

falling phase of the e.p.p. No such effect was observed. Therefore, the release of acetylcholine seems to end soon after the depolarizing current pulse.

Considering the complicated geometrical features of the junctional cleft, the decrease in transmitter concentration due to diffusion may be slower when the initial amount liberated is larger, possibly occupying more space in the junction. Diffusion of transmitter out of the junction region differs according to the geometry of the synapse (Eccles & Jaeger, 1958). The transmitter concentration at a circular synaptic contact ($2\ \mu$ in diameter) decreases to a half by 0.15 msec while at a long strip-like junction ($4\ \mu$ in width) by 2 msec. Therefore, the spatial factor may contribute to different time course of e.p.p. of various amplitudes, particularly if the spatial distribution of transmitter is different depending on the amount released. However, it seems that the falling phase of a large e.p.p., the half-decay time of which is more than 50 msec, is too slow to be explained by a diffusion process, even if the dimension of the junctional contact were 3 times as large as that assumed by Eccles & Jaeger (1958).

A possible explanation for the dependence of the time course of falling phase on the amplitude of e.p.p. is that, in the presence of prostigmine, concentrations of acetylcholine determine not only the magnitude but also the time course of an increase in conductance of the post-synaptic membrane. The mechanism underlying the prolongation of falling phase observed in the present experiments would be similar to that suggested to explain the action of xylocaine and its derivatives (Steinbach, 1968*a*). Steinbach (1968*b*) explained the prolongation of e.p.p. with xylocaine by assuming a continuous activity of the acetylcholine-receptor-drug complex which produces a prolonged increase in permeability of the post-synaptic membrane. D-Tubocurarine may affect the reaction of the complex production and shorten the falling phase of the e.p.p.

However, a difficulty in this hypothesis is that the time course of the response produced by iontophoretic application of acetylcholine was not prolonged as the amplitude was increased. The difference could mean that acetylcholine was applied mainly to extra-junctional receptors (Miledi, 1960) which might be qualitatively different from the junctional receptors involved in the production of the e.p.p. elicited by depolarization of the nerve terminal, or that the acetylcholine potentials are so much slower than the e.p.p. that their time courses are not strictly comparable. A similar problem arises with atropine which shortens the time course of the e.p.p. while it has no effect on the acetylcholine-potential evoked by iontophoretic application (Beránek & Vyskočil, 1968; Kordaš, 1968).

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