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FACTORS AFFECTING THE TIME COURSE OF DECAY OF END-PLATE CURRENTS: A POSSIBLE COOPERATIVE ACTION OF ACETYLCHOLINE ON RECEPTORS AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. End-plate currents have been studied in glycerol-treated frog sartorius nerve-muscle preparations with the voltage-clamp technique.

2. Adding the anticholinesterase prostigmine (3 μM) to the solution bathing the muscle caused a 2–7 (mean 3·3) times increase in the time constant of decay of end-plate currents. The anticholinesterase edrophonium (15 μM) also prolonged the time course of end-plate currents.

3. Pre-treatment of the preparation with collagenase, which leads to the removal of acetylcholinesterase in the synaptic cleft, prolongs the time course of end-plate currents.

4. Curare (1–2 μM), cobratoxin (0·13 μM), or α-bungarotoxin (0·13–0·26 μM) decreased the time constant of decay of end-plate currents in the presence of prostigmine.

5. These observations are consistent with the suggestion that repeated binding of acetylcholine (ACh) molecules to receptors as the ACh escapes from the synaptic cleft can contribute to the prolongation of end-plate currents which occurs when acetylcholinesterase activity is eliminated.

6. Increasing the amount of transmitter released from the presynaptic nerve terminal leads to a prolongation of end-plate currents in the presence of prostigmine.

7. In the presence of prostigmine, the second of two end-plate currents (interval 2–10 msec) decays more slowly than the first.

8. ACh (1–40 μM) or carbachol (40 μM) applied in the solution bathing the muscle prolongs end-plate currents in the presence of prostigmine.

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9. It is suggested on the basis of the observations described in paragraphs 6 to 8 that the time constant of decay of end-plate currents in the presence of prostigmine increases with increasing concentrations of ACh in the synaptic cleft. In the absence of prostigmine, increasing the concentration of ACh in the synaptic cleft did not change the time constant for decay of end-plate currents.

10 We interpret these results to suggest that ACh can have a *cooperative* action on receptors such that the association of ACh with one receptor (defined as binding a single ACh molecule) favours the *binding* or *retention* of ACh at other receptors. This implies that receptors can interact.

INTRODUCTION

It is well known that anticholinesterases prolong the durations of end-plate potentials (Feng, 1940; Eccles, Katz & Kuffler, 1941, 1942; Eccles & MacFarlane, 1949; Fatt & Katz, 1951) and of end-plate currents (Takeuchi & Takeuchi, 1959; Kordaš, 1968*a*, 1972; Magleby & Stevens, 1972*a*; Kuba, Albuquerque & Barnard, 1973). It has been argued (Eccles & Jaeger, 1958; Kuba & Tomita, 1971; Magleby & Stevens, 1972*a*) that free diffusion of acetylcholine (ACh) from the synaptic cleft would be too rapid to account for the prolonged time course of end-plate potentials and end-plate currents recorded in the presence of an anticholinesterase which inhibits the hydrolysis of ACh. This has led to the suggestion that the prolonging effect of the anticholinesterase prostigmine on end-plate potentials and end-plate currents might arise from an increase in the mean lifetimes of conductance channels activated by ACh (Kuba & Tomita, 1971; Magleby & Stevens, 1972*a*). However, Katz & Miledi (1973*a*) have reported that prostigmine has little or no effect on channel lifetime and have suggested that in the presence of prostigmine the action of ACh can be prolonged by delayed diffusion of ACh from the synaptic cleft resulting from repeated binding of ACh to receptors.

The purpose of the present paper is to further analyse factors affecting the decay of end-plate currents by using the voltage-clamp technique.

In the first part of this paper we present experiments showing that blocking acetylcholinesterase by prostigmine, edrophonium or collagenase leads to a prolongation of end-plate currents. Curare, cobratoxin and α-bungarotoxin are found to reduce the prolongation of end-plate currents caused by prostigmine. These results suggest that repeated binding of ACh to receptors can contribute to the prolongation of end-plate currents in prostigmine.

In the second part of this paper we present data which indicate that the time constant of decay of end-plate currents in the presence of prostig-

mine increases with increasing concentrations of ACh in the synaptic cleft. We interpret these results in terms of a cooperative action of ACh on post-synaptic receptors.

Some of these results have appeared previously in abstract form (Terrar & Magleby, 1974).

METHODS

The results reported here are based upon observations made on more than 150 myoneural junctions in over forty separate nerve-muscle preparations from the frog *Rana pipiens*. Experiments were done throughout the year at room temperature (20–24° C).

The standard bathing solution used in these experiments had the composition (mM): NaCl, 116; KCl, 2·0; $CaCl_2$, 1·8; Na_2HPO_4, 1·92; NaH_2PO_4, 0·48. The drugs used in these experiments, AChI (Sigma), carbachol (Carbamoylcholine chloride, Sigma), prostigmine (neostigmine methylsulphate, Sigma), curare (D-tubocurarine chloride, Sigma), and edrophonium (Roche) were prepared fresh daily and added to the bathing solution. All solutions were adjusted to pH 7·2–7·4 before use. In experiments where prostigmine was used the concentration was 3 μM unless otherwise specified, and the preparation was always exposed to this concentration for at least 30 min before starting to experiment. The cobratoxin (*siamensis* 3, Karlsson, Arnberg & Eaker, 1971) and α-bungarotoxin were gifts from R. Chang, T. H. Chiu, and L. T. Potter.

To prevent muscle contraction produced by nerve stimulation muscles were pre-treated with hypertonic glycerol (Gage & Eisenberg, 1967; Howell, 1969) by bathing the nerve-muscle preparation for about 60 min in the standard bathing solution to which 500–900 mM glycerol had been added. The muscles were then transferred to the standard bathing solution and allowed to recover for 30–60 min before use. Resting potentials of surface fibres used in this study typically ranged from -20 to -50 mV. An effort was not made to obtain muscle fibres with more normal resting potentials since these fibres usually had action potentials which propagated into the region from which end-plate currents were recorded from their presumed site of origin at the end-plate at the other end of the muscle.

The voltage-clamping apparatus used here is similar to that described previously (Connor & Stevens, 1971; Magleby & Stevens, 1972*a*). Under voltage-clamp conditions, the maximum membrane potential variations at the site of the voltage electrode during the end-plate current were less than 2–5 % of the difference between the equilibrium potential and the holding potentials. Voltage recording and current micro-electrodes were filled with 3 M-KCl and had resistances ranging from 2 to 10 MΩ (measured with 1 nA of current in the bathing solution). Voltage and current data were recorded on FM tape system. The data were later photographed from an oscilloscope with a Polaroid or kymograph camera, enlarged and measured by hand. Time constants characterizing the decay of end-plate currents were determined from semilogarithmic plots of the data or from the slopes of lines generated by a logarithmic amplifier (Analog Devices, 755P). The DC level of the signal fed into the logarithmic amplifier was continuously monitored to prevent errors resulting from possible base-line shifts. The logarithmic amplifier was also used during the experiments to give immediate information on the time constant of decay of end-plate currents. Similar results were obtained either from hand measured data or from data processed by the logarithmic amplifier.

End-plates were localized by the presence of fine nerve terminals. The voltage and current electrodes were then placed within 50–100 μm of each other at the region

of the muscle fibre which gave the maximum amplitude end-plate potentials or currents. This position was determined by moving the electrodes along muscle fibres.

The preparation chamber was continuously perfused with bathing solution in the experiments which examined the effects of various drugs on end-plate currents. Solutions were changed by switching the perfusion fluid. The electrodes were kept in the muscle fibre during the solution changes. The drift in the recording electrode throughout acceptable experiments did not exceed 3 mV.

The time constant of decay of end-plate currents is a function of the membrane potential (Kordaš, 1969; Magleby & Stevens, 1972*a*, *b*). Although we did not make a systematic examination of the effect of voltage on the time course of end-plate currents under the different experimental conditions used in this study, we did find that each of the observations reported in this paper could be observed over a wide range of holding potentials (usually +60 to −120 mV).

RESULTS

PART I

End-plate currents decay slower in the presence of prostigmine

Fig. 1 illustrates the effect of 3 μM prostigmine on the time course of end-plate currents recorded at the neuromuscular junction of a frog skeletal muscle fibre. End-plate currents are shown before (Fig. 1*A*) and 21 min after (Fig. 1*B*) prostigmine was applied in the solution flowing over the muscle. Semilogarithmic plots of the decay of these end-plate currents are shown in Fig. 1*C* where it can be seen that prostigmine caused a 2·2 fold prolongation of the decay of end-plate currents, the time constant for decay increasing from 1·4 to 3·1 msec. This effect of prostigmine acting to prolong end-plate currents was always seen, and in a series of experiments the mean time constant for decay of end-plate currents increased from $1{\cdot}35 \pm 0{\cdot}22$ msec (mean ± S.D. of an observation, $n = 14$) in normal solution to $4{\cdot}49 \pm 1{\cdot}70$ msec ($n = 15$) in the presence of prostigmine, the mean increase being 3·3 times. The fibres selected for this comparison had membrane potentials (holding potentials) within ±20 mV of −60 mV and were exposed to prostigmine for more than 30 min. The effect of prostigmine in prolonging end-plate currents (Takeuchi & Takeuchi, 1959; Kordaš, 1968*a*, 1972; Magleby & Stevens, 1972*a*, *b*) and end-plate potentials (e.p.p.s) (Eccles & MacFarlane, 1949; Fatt & Katz, 1951; Kuba & Tomita, 1971) is well known. Prostigmine was used at 3 μM because this concentration has been shown to effectively eliminate (block 97% of) cholinesterase activity (Wilson, 1955; Levin & Jandorf, 1955). Higher doses have a curare-like effect (Katz & Miledi, 1973*a*).

Edrophonium and collagenase treatment also prolong end-plate currents

If the effect of prostigmine in prolonging end-plate currents is a consequence of its anticholinesterase activity then reducing the acetylcholin-

esterase activity by other means should also result in a similar prolongation. The effects of the fast-acting anticholinesterase edrophonium and of collagenase treatment (which results in an irreversible loss of esterase activity (Betz & Sackman, 1971, 1973; Hall & Kelly, 1971)) were therefore examined.

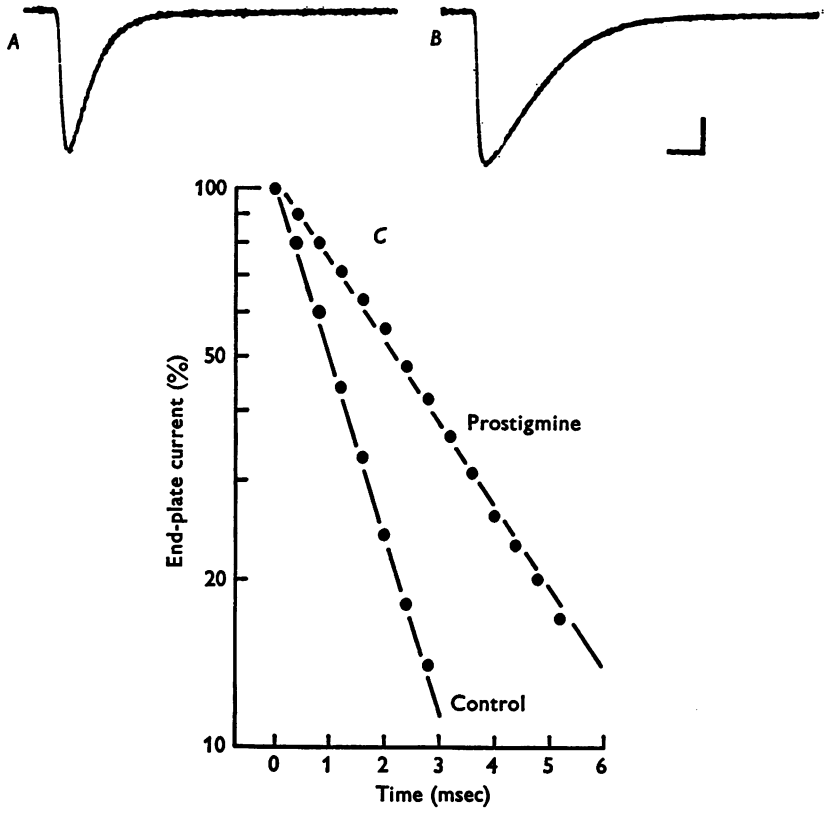

Fig. 1. Effect of prostigmine on the time course of end-plate currents recorded under voltage clamp from the frog sartorius nerve-muscle preparation. *A*, end-plate current recorded in normal bathing solution. *B*, end-plate current recorded from the same fibre 21 min after adding 3 μM prostigmine to the bathing solution flowing over the muscle. *C*, decay of the end-plate currents shown in *A* and *B* plotted semilogarithmically as a function of time. The time constant of decay (τ) increased from 1·4 to 3·1 msec after exposure to prostigmine. Vertical bar: 200 nA. Horizontal bar: 2 msec. Membrane potential (holding potential): -113 mV.

Fig. 2 illustrates end-plate currents recorded during exposure to 15 μM edrophonium applied in a flowing solution bathing the muscle. Fig. 2*A* shows a record before the edrophonium was applied, and Fig. 2*B*, *C*, and *D* show end-plate currents recorded 1·4, 3·5, and 13 min after the edrophonium

first flowed into the bath. Semilogarithmic plots of the decays of these currents are shown in Fig. 2*E*. It can be seen that there was a graded increase in the time constant of decay with increasing duration of exposure

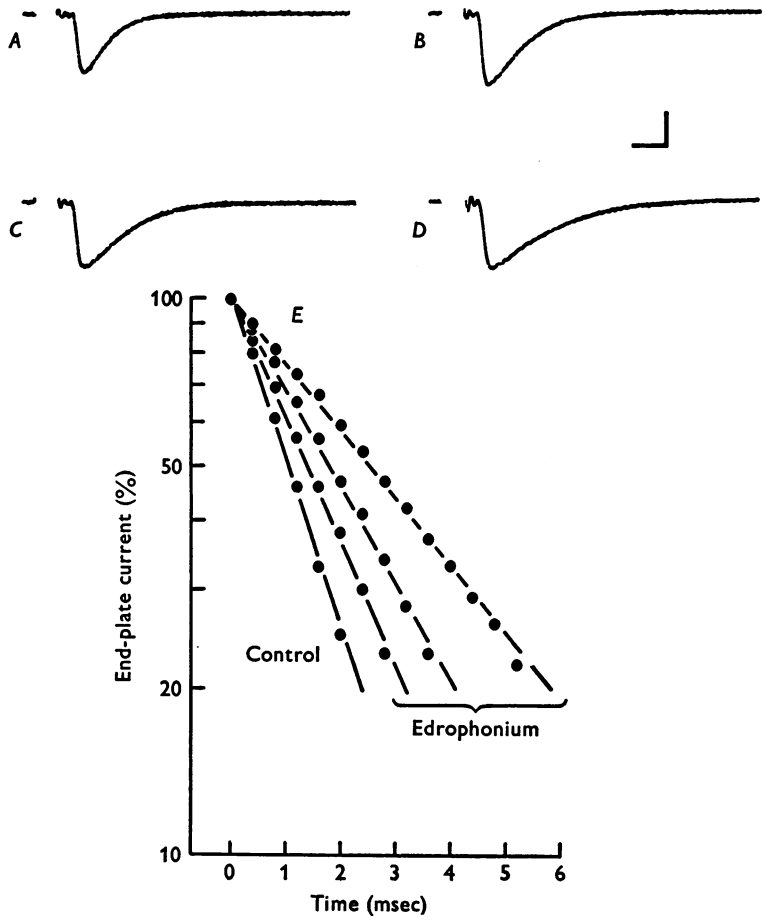

Fig. 2. Effect of edrophonium on the time course of end-plate currents recorded from a single muscle fibre before (*A*), and after 1·4 min (*B*), 3·5 min (*C*) and 13 min (D), exposure to 15 μM edrophonium. *E*, decays of end-plate currents shown in *A*–*D* plotted semilogarithmically against time. τ: *A*, 1·5 msec; *B*, 2·0 msec; *C*, 2·6 msec; *D*, 3·6 msec. Vertical bar: 200 nA. Horizontal bar: 2 msec. Holding potential: −38 mV.

to edrophonium presumably arising from an increasing blockade of the acetylcholinesterase. The effect of edrophonium in prolonging end-plate currents was always seen and was rapidly reversible.

Fig. 3 shows end-plate currents in two typical preparations before

(Fig. 3*A*, *B*) and after (Fig. 3*C*, *D*) collagenase (Sigma type I) treatment (0·01 % for 60–120 min). As was the case when the acetylcholinesterase was blocked with prostigmine or edrophonium, end-plate currents in

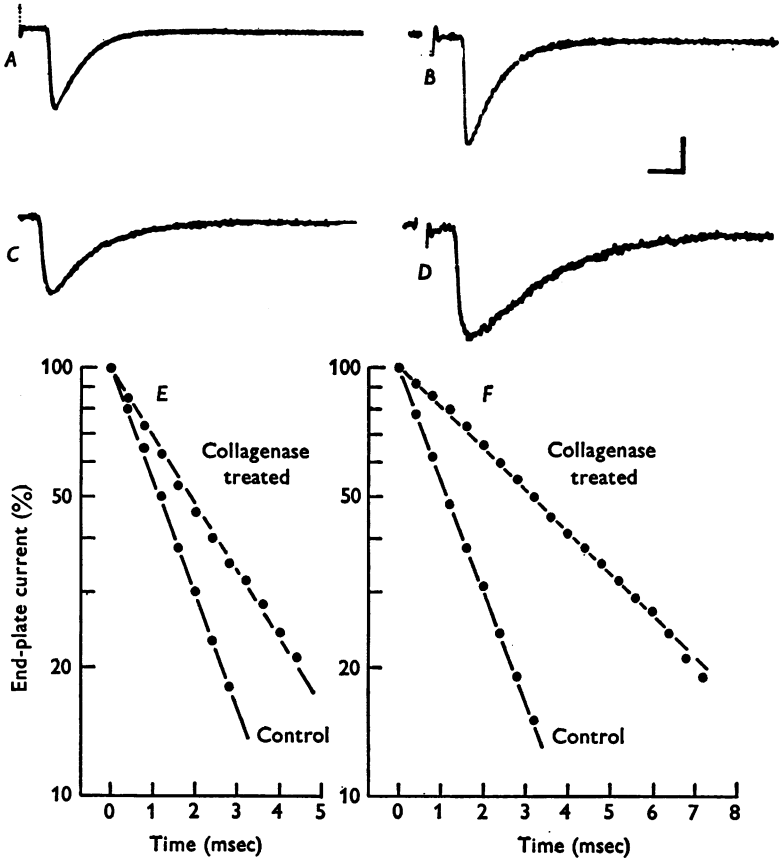

Fig. 3. Effect of pre-treatment with collagenase on the time course of end-plate currents. *A* and *B* recorded before, and *C* and *D* after collagenase treatment (0·01 % for 60–120 min). *E* and *F*, decays of end-plate currents plotted semilogarithmically against time for one preparation shown in *A* and *C*, and for another in *B* and *D*. τ: *A*, 1·6 msec; *C*, 2·7 msec; *B*, 1·6 msec; *D*, 4·4 msec. Vertical bar: *A*, 100 nA; *C*, 80 nA; *B*, 80 nA; *D*, 40 nA. Horizontal bar: 2 msec. Holding potentials: *A*, −72 mV; *C*, −70 mV; *B*, −82 mV; *D*, −75 mV.

collagenase-treated muscle decayed more slowly than currents recorded from the untreated muscle. This is clearly shown by the semilogarithmic plots in Fig. 3*E* and *F* where end-plate currents decayed 1·7 and 2·8 times slower after collagenase treatment.

Consistent with the observations shown in Fig. 3 that end-plate currents are prolonged following collagenase treatment are the earlier findings of Albuquerque, Sokall, Sonesson & Thesleff (1968), Betz & Sackman (1971, 1973) and Hall & Kelly (1971) that collagenase treatment led to a prolongation of end-plate potentials, as would be expected if the underlying end-plate currents persisted longer, though it should be mentioned that end-plate potentials do not give a direct measure of the underlying conductance change because of the passive electrical properties of the muscle fibre (see Fatt & Katz, 1951).

The finding that three methods of inactivating acetylcholinesterase (prostigmine, edrophonium, and collagenase treatment) all lead to a marked lengthening of the time course of end-plate currents suggests that the anticholinesterase activity of these agents is an important factor contributing to their effect. Supporting this conclusion are the observations that blocking acetylcholinesterase by two additional methods, pretreatment with diisopropylfluorophosphate (Kuba *et al.* 1973; K. L. Magleby & D. A. Terrar, unpublished observations) and bath applied eserine (Takeuchi & Takeuchi, 1959), also prolong end-plate currents.

A quantitative comparison of the effects of the anticholinesterases and of the collagenase treatment has not been attempted because the edrophonium or collagenase treatments may not have inactivated all of the functional esterase. High concentrations of edrophonium were avoided (because of a possible direct effect on ACh receptors (Katz & Thesleff, 1957)) with the result that inhibition of esterase and the subsequent change in decay times may not have been maximal. Similarly it seems possible that the collagenase treatment may not have removed all the functional esterase, for example from fibres where the end-plate was not on the surface. If the muscle was exposed to collagenase for more than 1–2 hr in an attempt to give sufficient time for complete esterase removal, end-plate currents were usually reduced in amplitude with slow rising phases suggesting that the nerve terminal may have partially separated from the muscle fibre (Betz & Sackman, 1973).

Curare, cobratoxin, and α-bungarotoxin reduce the prolongation of end-plate currents in prostigmine

When acetylcholinesterase is inhibited by prostigmine, ACh is no longer rapidly hydrolysed, and consequently ACh must be removed from the synaptic cleft by diffusion. Katz & Miledi (1973*a*) have suggested that an important factor which can contribute to the prolongation of end-plate currents in the presence of prostigmine is the impairment to diffusion of ACh caused by its binding to receptors (delayed diffusion hypothesis). They postulated that ACh repeatedly binds to receptors along its route of escape as it diffuses from the synaptic cleft. If each ACh molecule makes m successful bindings as it escapes, each lasting t msec, then the time required for an ACh molecule to diffuse from the synaptic cleft is prolonged by an additional mt msec leading to a prolongation of end-plate currents.

An experimental test of the hypothesis that diffusion of ACh is delayed by its binding to receptors as it diffuses from the synaptic cleft would be to reduce the number of free receptors so that ACh would make fewer bindings and be delayed less as it escapes from the cleft.

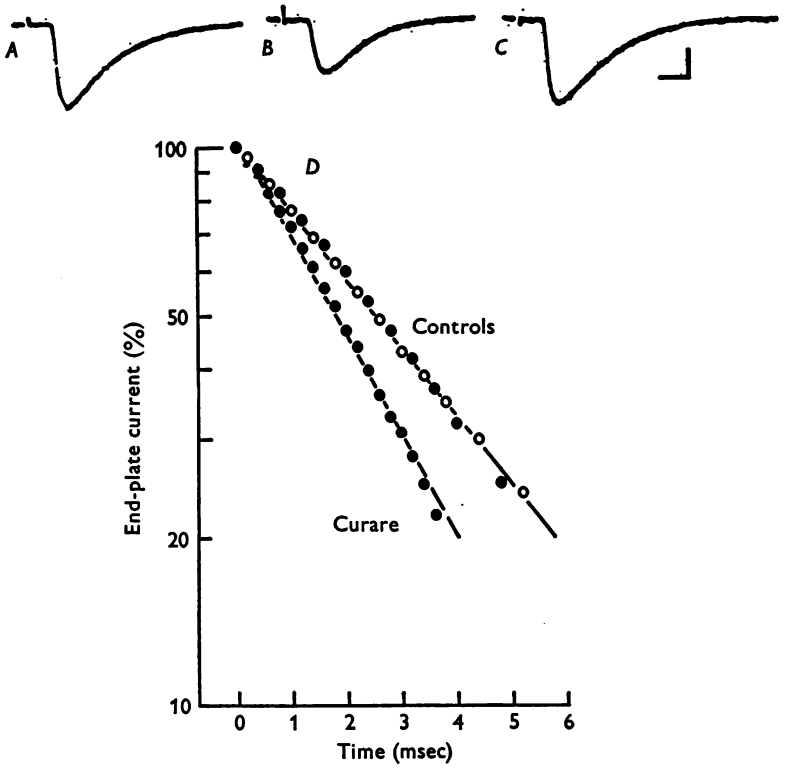

Fig. 4. Effect of curare on the time course of end-plate currents recorded in the presence of prostigmine. Records from a single muscle fibre before (*A*), during 1 μM curare (*B*), and after washing curate from the muscle (*C*). *D*, decays of end-plate currents shown in *A*–*C* plotted semilogarithmically against time. Open circles: after washing curare from muscle. τ: controls (*A* and *C*) 3·3 msec; in curare (*B*) 2·4 msec. Vertical bar: 80 nA. Horizontal bar: 2 msec. Holding potential: −95 mV.

The number of free receptors is expected to be reduced by curare. Therefore, if the above hypothesis is correct, curare would be expected to reduce the time constant of decay of end-plate currents in prostigmine. The effect of curare on the time course of end-plate currents recorded in prostigmine from a typical experiment is illustrated in Fig. 4 which shows records taken from a single muscle fibre before (*A*), during (*B*) and after (*C*) 1 μM curare was applied in the solution bathing the muscle.

Semi-logarithmic plots of the decays are shown in Fig. 4*D*. It can be seen that curare increased the rate of decline of end-plate currents. This effect was always seen at the examined concentrations of curare of 1–2 μM and was reversible.

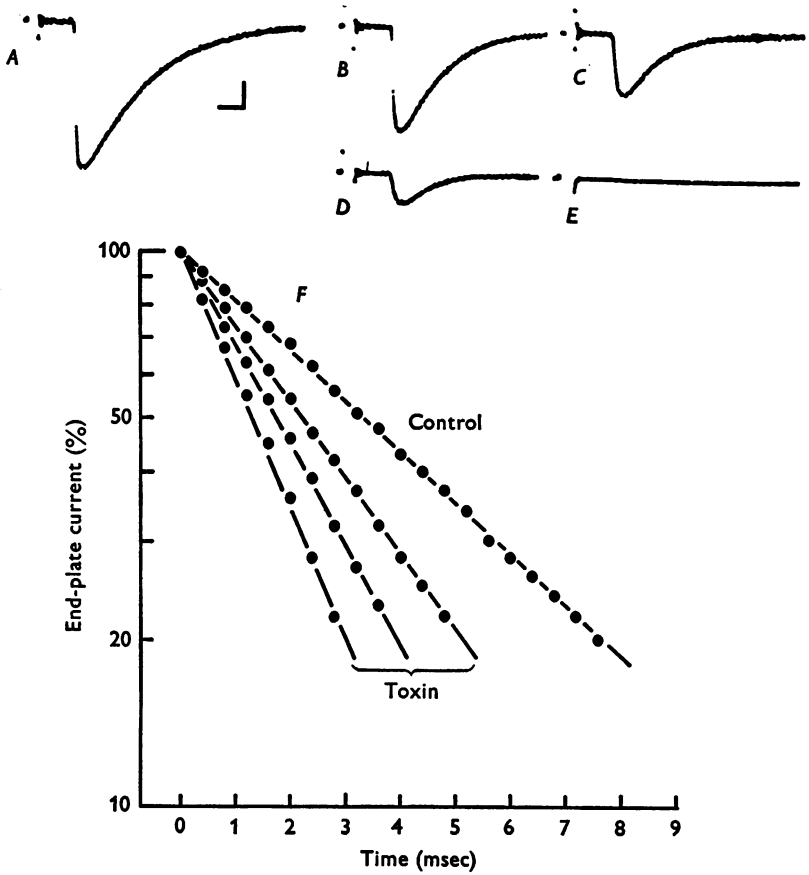

Fig. 5. Effect of cobratoxin on the time course of end-plate currents recorded in the presence of prostigmine. Records from a single muscle fibre before (*A*) and during increasing durations of exposure (*B–E*) to 0·13 μM toxin. *F*, decays of end-plate currents shown in *A–E* plotted semi-logarithmically against time. τ: *A*, 4·8 msec; *B*, 3·2 msec; *C*, 2·5 msec; *D*, 1·9 msec. Vertical bar: 80 nA. Horizontal bar: 2 msec. Holding potential: −38 mV.

Consistent with these data are the findings (Katz & Miledi, 1973*a*) that curare decreases the half-time of decay of externally recorded miniature end-plate potentials in the presence of prostigmine, and that increasing the curare concentration in a curare-blocked preparation decreases the half

time of decline of end-plate currents in the presence of prostigmine (Kordaš, 1968*b*). Curare has also been shown to counteract the prolongation of end-plate potentials which occurs in the presence of acetylcholinesterase inhibitors (Eccles, Katz & Kuffler, 1942; Fatt & Katz, 1951; Beránek & Vyskočil, 1968).

Another method of reducing the number of free receptors is to apply cobratoxin (from *Naja naja siamensis*) which binds specifically to post-synaptic receptors (Chang & Lee, 1966; Lester, 1972*a*), its effect being only very slowly reversible. Fig. 5*A* shows an end-plate current recorded in prostigmine before the toxin was applied. The records in Fig. 5*B–E* were recorded successively over a 15 min period of exposure to the toxin (1 μg/ml., 0·13 μM) applied in the solution bathing the muscle. The time constant of end-plate currents became faster as the amplitude of end-plate currents was reduced by the toxin. This progressive decrease in the time constant to decay of end-plate currents is clearly shown in Fig. 5*F* where the decays are plotted semilogarithmically.

In a series of experiments similar to that shown in Fig. 5, it was found that the time constant of decay of end-plate currents in prostigmine was also decreased by bath application of α-bungarotoxin (1–2 μg/ml., 0·13–0·26 μM) which blocks receptors (Chang & Lee, 1963; Miledi & Potter, 1971). An example of such an experiment is shown in Fig. 6 where the time constants of decay of end-plate currents are plotted against their peak amplitudes. It can be seen that the time constants for decay of end-plate currents were reduced as the peak end-plate currents decreased, presumably because of a progressive blocking of receptors by the toxin. Consistent with these observations is the finding of Katz & Miledi (1973*a*) that α-bungarotoxin decreases the half-time of decay of externally recorded miniature end-plate potentials in the presence of prostigmine. Notice from Fig. 6 that the time constant of decay is not linearly related to the peak end-plate current as might be expected (1) if m, the number of repeated bindings of ACh molecules to receptors as it escapes from the synaptic cleft (and consequently the time constant of decay of end-plate currents) is proportional to the number of free receptors not blocked by the toxin, and (2) if the peak end-plate current is proportional to the number of free receptors. Experiments with cobratoxin showed a similar non-linearity. A possible reason for this non-linearity will be discussed later.

The decrease in time constant of decay of end-plate currents which is caused by curare or toxin in the presence of prostigmine supports the hypothesis that diffusion of ACh out of the synaptic cleft can be delayed by its binding to receptors. It should be emphasized that curare has little or no effect on the time course of end-plate currents in the absence of prostigmine when functional esterase is present (Kordaš, 1968*b*; Magleby &

Stevens, 1972*a*). The removal of ACh by hydrolysis is apparently so rapid when functional esterase is present that there is little or no chance for repeated binding of ACh to receptors, and in this case the time course of end-plate currents is determined by the lifetimes of conductance channels (Magleby & Stevens, 1972*a*, *b*; Anderson & Stevens, 1973).

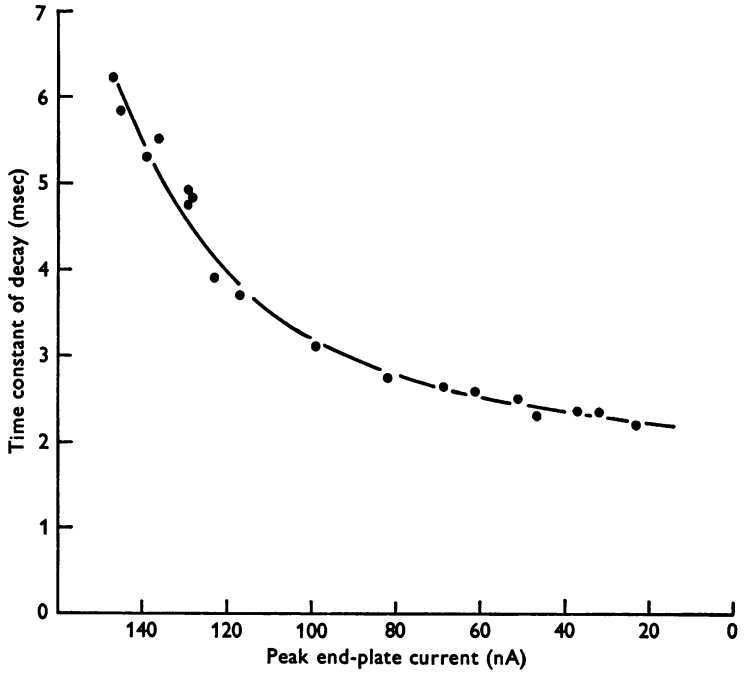

Fig. 6. Time constant of decay of end-plate current as a function of peak end-plate current during exposure to 0·26 μM α-bungarotoxin in the presence of prostigmine. The first point (τ = 6·2 msec) was obtained before toxin flowed into the muscle chamber. Holding potential: −42 mV.

Estimation of p, the fraction of ACh bound

Katz & Miledi (1973*a*) suggested that if a fraction p of the released ACh molecules were bound to receptors, diffusion from the synaptic cleft would be slowed by the factor $1/(1-p)$ relative to the case of free diffusion, where $p = 0$. They calculated the fraction p, from

$$p = \frac{t_1 - t_2}{t_1 - vt_2}, \tag{1}$$

where t_1 is the time constant of decay before curate or toxin, t_2 is the time constant during curare or toxin, and v is the factor by which p is reduced in the presence of curare or toxin. An estimate of v was obtained from the ratio of the amplitude of end-plate currents during curare or toxin to the

amplitude before the drug was applied. Applying equation (1) to data like that shown in Figs. 4 and 5 obtained using curare, cobratoxin or α-bungarotoxin we find p to be $0{\cdot}58 \pm 0{\cdot}06$ (mean $\pm$ S.D., $n = 13$). These values of p, which we found for end-plate currents, are similar to those found by Katz & Miledi (1973*a*) from externally recorded miniature end-plate potentials which result from single quanta. Katz & Miledi suggested that these values of p implied that a large fraction (about 60 %) of released ACh is bound to receptors.

Ionophoresis of ACh molecules is not an important factor determining the rate of decay of end-plate currents in prostigmine

When the membrane potential is more negative than the equilibrium potential for the end-plate current, the end-plate current will flow through the synaptic cleft into the muscle fibre at the neuromuscular junction. Since ACh bears a positive charge it might be thought that the inward end-plate current could oppose the diffusional loss of ACh from the synaptic cleft by an ionophoretic effect. Conversely when the end-plate current flows outward from the post-synaptic membrane at membrane potentials more positive than the equilibrium potential this ionophoretic effect would tend to facilitate the removal of ACh from the synaptic cleft. Reducing the magnitude of end-plate currents might therefore be expected to increase the rate of decay of end-plate currents which are directed inwards (at membrane potentials more negative than the equilibrium potential) and conversely to decrease the rate of decay of end-plate currents which are directed outwards (at membrane potentials more positive than the equilibrium potential). Since curare (or toxin) reduced the magnitude of end-plate currents, it might be thought that at membrane potentials more negative than the equilibrium potential, curare could increase the rate of decay of end-plate currents in prostigmine (1) by reducing the ionophoretic effect which tends to oppose diffusional loss of ACh, and (2) by receptor occlusion as suggested previously. However, when the ionophoretic influence on ACh was reversed by holding the membrane potential positive to the equilibrium potential, curare still decreased the rate of decay of end-plate currents, suggesting that reducing the number of free receptors (so that ACh would make fewer bindings and by delayed less as it diffuses from the synaptic cleft) is the more important contributory factor. An estimate of the contribution of the ionophoretic effect can be made by comparing estimates of p, the fraction of ACh bound, at different membrane potentials. If ionophoresis of ACh by end-plate currents is significant, this fraction p should be over-estimated at more negative membrane potentials (when reducing the ionophoretic effect would quicken the decay of end-plate currents) and underestimated at more positive membrane

potentials. Values of p were found not to be dependent on membrane potential suggesting that ionophoresis of ACh has little or no influence on the time course of decay of end-plate currents. Values of p obtained by applying equation (1) to currents recorded at a series of membrane potentials before and during the application of 2 μM curare in the solution bathing the muscle are: resting potential, $P = 0{\cdot}56$, range = 0·49–0·65; hyperpolarized by 40 mV, $P = 0{\cdot}57$, range = 0·53–0·61; depolarized by 40 mV, $P = 0{\cdot}57$, range = 0·50–0·64 ($n = 3$).

PART II

The data presented in the first part of this paper are consistent with the suggestion that repeated binding of ACh molecules to receptors can contribute to the prolongation of end-plate currents which occurs in the presence of prostigmine. The binding of ACh to receptors leading to activation of conductance channels has usually been considered in terms of individual ACh molecules binding to independent, non-interacting receptors (del Castillo & Katz, 1957; Magleby & Stevens, 1972*b*; Kordaš, 1972). (We define a receptor as capable of binding a single ACh molecule.) If these models apply in the presence of prostigmine, the time constant of decay of end-plate currents would not be expected to be influenced by the amount of transmitter released from the nerve terminal or by the concentration of ACh in the synaptic cleft. In this section we present evidence from three different experimental approaches to suggest that the time constant of decay of end-plate currents in prostigmine does increase with the concentration of ACh in the synaptic cleft.

Increasing the amount of transmitter released from the nerve terminal leads to a prolongation of end-plate currents in prostigmine

One way of changing the amount of transmitter released from the nerve terminal is to exploit the variations in quantity of transmitter released which result during repetitive stimulation. With prolonged rapid repetitive stimulation, the amount of transmitter released from the presynaptic nerve terminal decreases (del Castillo & Katz, 1954*a*; Lass, Halevi, Landau & Gitter, 1973). Fig. 7*A* shows end-plate currents in prostigmine recorded at two levels of transmitter release. The gain was reduced to record the larger currents (indicated by arrow) which were recorded while stimulating once every 2 sec. Transmitter release was then reduced by stimulating at 10/sec until the amplitudes of the end-plate currents were reduced by half, when the second pair of end-plate currents was recorded. Semilogarithmic plots of these currents are shown in Fig. 7*C* as filled circles (large currents) and as filled squares (small currents). It is clear from Fig. 7*A* and *C* that slower rates of decay were associated with larger currents. This result that end-

plate currents decayed faster when transmitter release was reduced in prostigmine was always seen. A possible complicating factor in interpreting these results is that repetitive stimulation might lead to a desensitization of the post-synaptic membrane (Thesleff, 1959; but see Otsuka, Endo & Nonomura, 1962; and Bowen, 1972). However, we found that end-plate currents recorded in the presence of prostigmine decayed slower when transmitter release was increased by either post-tetanic potentiation or facilitation, both of which act to increase transmitter release (Hubbard, 1963; Magleby, 1973). This observation is consistent with the suggestion that the time constant of decay of end-plate currents in the presence of prostigmine varies with the amount of transmitter released from the nerve terminal. Another method of varying transmitter release is to change the calcium and magnesium concentrations in the external solution (del Castillo & Engbaek, 1954; Jenkinson, 1957). Using this method, Kordaš (1972) has shown that in the presence of prostigmine large end-plate currents decay more slowly than small end-plate currents.

A possible interpretation of these data is that in the presence of prostigmine the time constant of decay of end-plate currents increases with the concentration of ACh in the synaptic cleft. Although the concentration of ACh in the synaptic cleft is expected to be increased when more ACh is released from the nerve terminal, the concentration of ACh in the region of receptors is not expected to increase linearly with the amount of transmitter released from the nerve terminal because of the distributed nature of quantal transmitter release (del Castillo & Katz, 1954*b*, 1956). The effect of increasing transmitter release in prolonging end-plate currents is not primarily caused by an ionophoretic effect of the larger end-plate currents acting to retain ACh in the synaptic cleft since increasing transmitter release also led to a prolongation of end-plate currents at membrane potentials more positive than the equilibrium potential for the end-plate current when the influence of the ionophoretic effect would be reversed.

On the basis of the delayed diffusion hypothesis, the increase in the time constant for the decay of end-plate currents in the presence of prostigmine with increasing concentrations of ACh in the synaptic cleft could occur if there were an increase either in m, the number of successful bindings of ACh to receptors on its route of escape from the cleft, or in t, the time during which an ACh molecule is retained on each receptor. However, if receptors were independent and non-interacting an increase in synaptic cleft ACh concentration should not increase m or t. Therefore these data suggest that in the presence of prostigmine receptors are not independent and do interact. In other words, ACh acts *cooperatively* on receptors such that increasing the concentration of ACh in the synaptic cleft leads to an increase in m and/or t.

The decay of end-plate currents in the absence of prostigmine when acetylcholinesterase is functional is determined by the lifetimes of individual conductance channels (Magleby & Stevens, 1972*a*, *b*; Anderson & Stevens, 1973). If channel lifetime does not vary with the concentration of ACh in the synaptic cleft in the absence of prostigmine then varying the amount of transmitter released from the nerve terminal should not modify the rate of decay of end-plate currents in the absence of prostigmine. End-plate currents at high (recorded at reduced gain) and low levels of transmitter release are shown in Fig. 7*B* and their decays are plotted semilogarithmically in Fig. 7*C* as open circles (large currents) and open squares (small currents). This Figure shows that in the absence of prostigmine the time constant for decay of end-plate currents is independent of the amount of transmitter released from the nerve terminal.

In the presence of prostigmine the second of two end-plate currents decays more slowly than the first

In the experiment illustrated in Fig. 8*A* either single, or a pair (interval 5 msec) of stimuli were alternately applied to the motor nerve. The upper part of Fig. 8*A* shows end-plate currents while the lower part shows analogue derived semilogarithmic plots of the currents. This Figure shows that in the presence of prostigmine an end-plate current evoked 5 msec after another decayed more slowly than did an end-plate current evoked by a single shock to the motor nerve. This is more clearly demonstrated in the semilogarithmic plot of their decays shown in Fig. 8*C* (filled

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Fig. 7. Effect of the amount of transmitter released from the nerve terminal on the time course of end-plate currents.

A, in prostigmine. Four records of responses are shown, two at low amplification (indicated by arrow) and two at high (2 ×) amplification. The low amplification records were obtained while stimulating at once every 2 sec, while the high amplification records were obtained after stimulating at 10/sec until the end-plate currents were reduced to one half of their initial amplitude (because of reduced transmitter release).

B, without prostigmine and from a different preparation, otherwise same as *A*. Vertical bar: *A*, 40 nA, 20 nA; *B*, 50 nA, 25 nA. Horizontal bar: 2 msec. Holding potential: *A*, −70 mV; *B*, −32 mV. $[Ca^{2+}]$ was 3·6 mM in *A* and *B*.

C, decays of end-plate currents in *A* and *B* plotted semilogarithmically against time. Filled circles: large end-plate currents in *A*, τ = 6·3 msec. Filled squares: small end-plate currents in *A*, τ = 4·4 msec. Open circles: large end-plate currents in *B*, τ = 1·4 msec. Open squares: small end-plate currents in *B*, τ = 1·4 msec.

Notice that larger currents decay slower in the presence of prostigmine (arrow in *A*), while the decays appear independent of the end-plate current amplitude and hence of transmitter release in the absence of prostigmine (*B*).

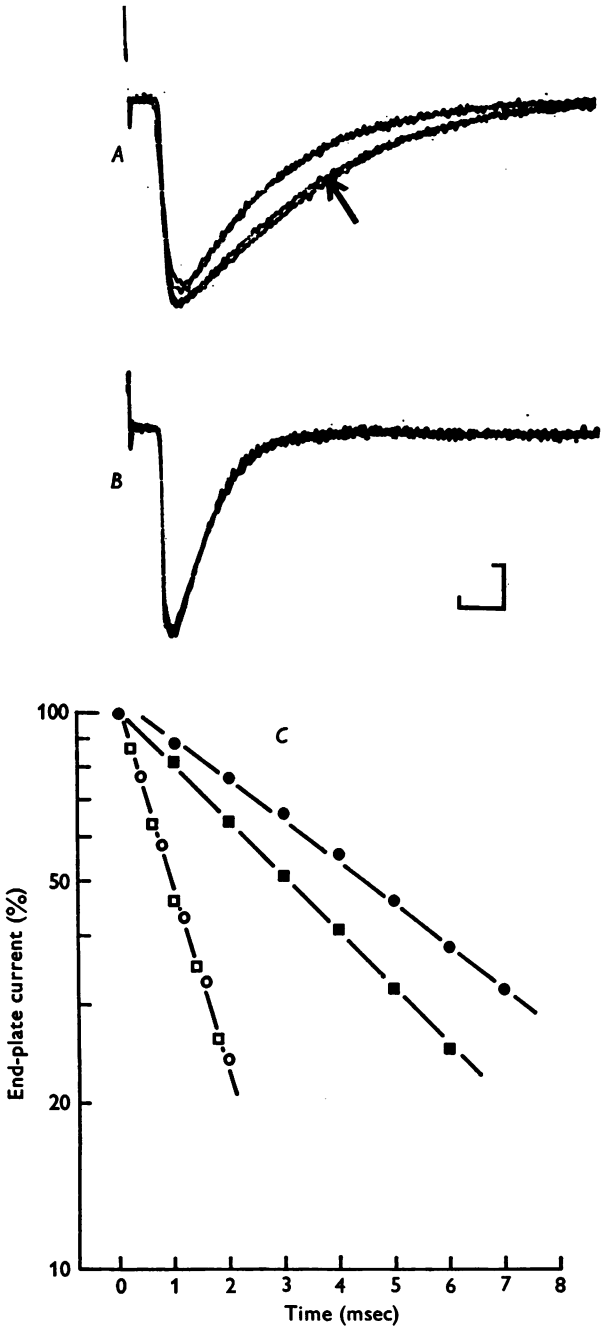

Fig. 7*A*, *B* and *C*. For legend see facing page.

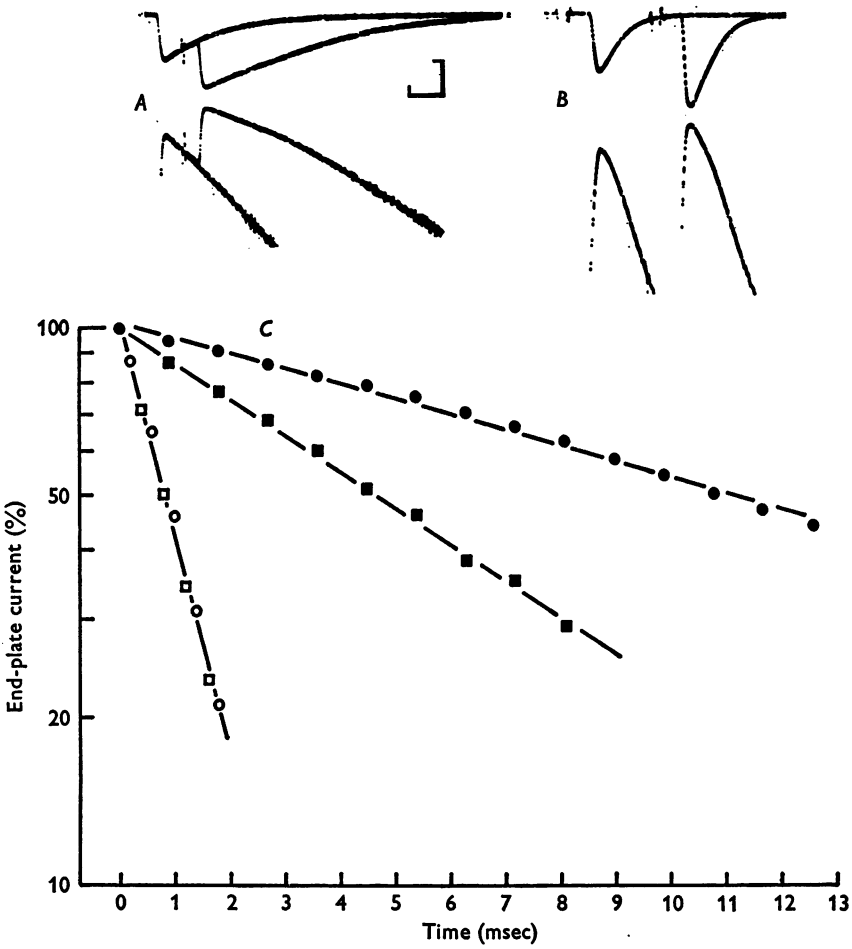

Fig. 8. Effect of a second nerve impulse on the time course of decay of end-plate currents.

A, in prostigmine. Superimposed records resulting from a single shock to the nerve and from a pair of shocks separated by a 5 msec interval. Upper traces: end-plate currents. Lower traces: analogue derived semilogarithmic plots of upper traces.

B, without prostigmine and from a different preparation, otherwise same as *A*. Vertical bar: 0·25 log units; 200 nA in *A* and *B*. Horizontal bar: *A*, 5 msec; *B*, 2 msec. Holding potential: *A*, −20 mV; *B*, −49 mV.

C, decays of end-plate currents in *A* and *B* plotted semilogarithmically against time. Filled squares: first end-plate current in *A*, τ = 6·3 msec. Filled circles: second end-plate current in *A*, τ = 15·7 msec. Open squares: first end-plate current in *B*, τ = 1·2 msec. Open circles: second end-plate current in *B*, τ = 1·2 msec.

Notice that the second end-plate current decays slower in prostigmine. In the absence of prostigmine the second end-plate current decays with the same time constant as the first, and this was also observed at shorter intervals when the second end-plate current fell on the first.

squares, first end-plate current; filled circles, second end-plate current. Similar observations have been reported by Kordaš (1972). It is expected that the concentration of ACh in the cleft is higher during the second of two end-plate currents both because of facilitated transmitter release (Mallart & Martin, 1967) and because some ACh remains from the previous release. These data, then, like those on variation of transmitter release from the nerve terminal, can be explained in terms of the postulate that in the presence of prostigmine the time constant of decay of end-plate currents increases with the concentration of ACh in the cleft. As expected from the data of Magleby & Stevens (1972*b*, p. 163), in the absence of prostigmine the second of two end-plate currents always decayed with the time constant similar to that of the first. This is shown by the experimental data in Fig. 8*B* which are plotted as open symbols in Fig. 8*C*.

Acetylcholine or carbachol applied in the solution bathing the muscle prolongs end-plate currents in prostigmine

The suggestion that in the presence of prostigmine end-plate currents can be further prolonged by increasing the concentration of ACh in the synaptic cleft can be experimentally tested by applying ACh in the solution bathing the muscle. Fig. 9*A* illustrates end-plate currents recorded from the same end-plate in prostigmine before and after (indicated by arrow) the concentration of ACh in the synaptic cleft was increased by bath application of 20 μM-ACh. It can be seen that the end-plate currents decayed more slowly in the presence of bath-applied ACh. This is shown by the semilogarithmic plots in Fig. 9*C* (filled squares, before ACh; filled circles, after ACh). In a series of similar experiments end-plate currents in prostigmine were prolonged by ACh at concentrations ranging from 1 to 40 μM, and by 40 μM carbachol. Records showing this prolongation were taken before desensitization became appreciable. A prolongation of end-plate currents by bath applied ACh was also seen at membrane potentials positive to the equilibrium potential for the end-plate currents again suggesting that ionophoresis of ACh by end-plate currents is not primarily responsible for the prolongation of end-plate currents which occurs in the presence of bath-applied ACh or carbachol. Supporting our suggestion that the decay of end-plate currents is a function of the concentration of ACh in the synaptic cleft is the observation of V. Dionne & C. F. Stevens (personal communication) that ionophoretically applied ACh prolongs the time course of miniature end-plate currents recorded in the presence of prostigmine.

In the absence of prostigmine neither ACh (1–60 μM) nor carbachol (40 μM) caused a significant prolongation of end-plate currents. An example of the lack of prolonging effect of 20 μM ACh on the time course of

end-plate currents in the absence of prostigmine is illustrated in Fig. 9*B*. The semilogarithmic plots of the decays of end-plate currents from Fig. 9*B* are shown in Fig. 9*C* (open symbols) where it can be seen that in the absence of prostigmine the time constant for decay of end-plate currents was similar before and during the application of 20 μM ACh. It is also clear from Fig. 9*B* and other similar experiments that in the absence of prostigmine bath applied ACh (1–60 μM) or carbachol (40 μM) does not markedly increase the amount or duration of transmitter release from the presynaptic nerve terminal, for if it did, the end-plate currents would not superimpose. It should be mentioned again that the data in Fig. 9 were obtained before desensitization became appreciable.

During prolonged applications of ACh or carbachol in the presence of prostigmine when desensitization of receptors might be expected to become appreciable (Fatt, 1950; Thesleff, 1955; Rang & Ritter, 1970; Jenkinson & Terrar, 1973) we found that the amplitude of end-plate currents began to decrease progressively and accompanying this decrease in amplitude was a reduction in the time constant of decay of end-plate currents. The time course of the effect of 20 μM ACh in first prolonging and then shortening the decays of end-plate currents in prostigmine is shown in Fig. 10 where the time constant of decay of end-plate currents is plotted against the duration of exposure to bath applied ACh. This decrease in the time constant of decay of end-plate currents which occurred after prolonged exposures to ACh or carbachol could arise from desensitization and perhaps also from a possible reduction in the amount of transmitter released. It was shown in Fig. 7 that decreasing transmitter release in the presence of prostigmine led to a quickening of end-plate currents, and bath-applied ACh or carbachol have both been reported to decrease transmitter release (Ciani & Edwards, 1963; Lester, 1972*b*). Desensitization could lead to a

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Fig. 9. Effect of bath applied ACh on the time course of end-plate currents.

A, with prostigmine. Four records are shown, two before and two after (indicated by arrow) exposure to 20 μM ACh applied in a flowing solution bathing the muscle.

B, without prostigmine and from a different preparation, otherwise same as *A*. Vertical bar: *A*, 17 nA; *B*, 40 nA. Horizontal bar: 2 msec. Holding potential: *A*, −47 mV; *B*, −49 mV.

C, decays of end-plate currents shown in *A* and *B* plotted semilogarithmically against time. Filled squares: before ACh in *A*, τ = 2·9 msec. Filled circles: during ACh in *A*, τ = 5·7 msec. Open squares: before ACh in *B*, τ = 1·2 msec. Open circles: during ACh in *B*, τ = 1·2 msec.

Notice that bath applied ACh prolonged end-plate currents in prostigmine (*A*) while in the absence of prostigmine all four currents superimpose (*B*). End-plate currents in the presence of bath applied ACh were recorded before desensitization became appreciable.

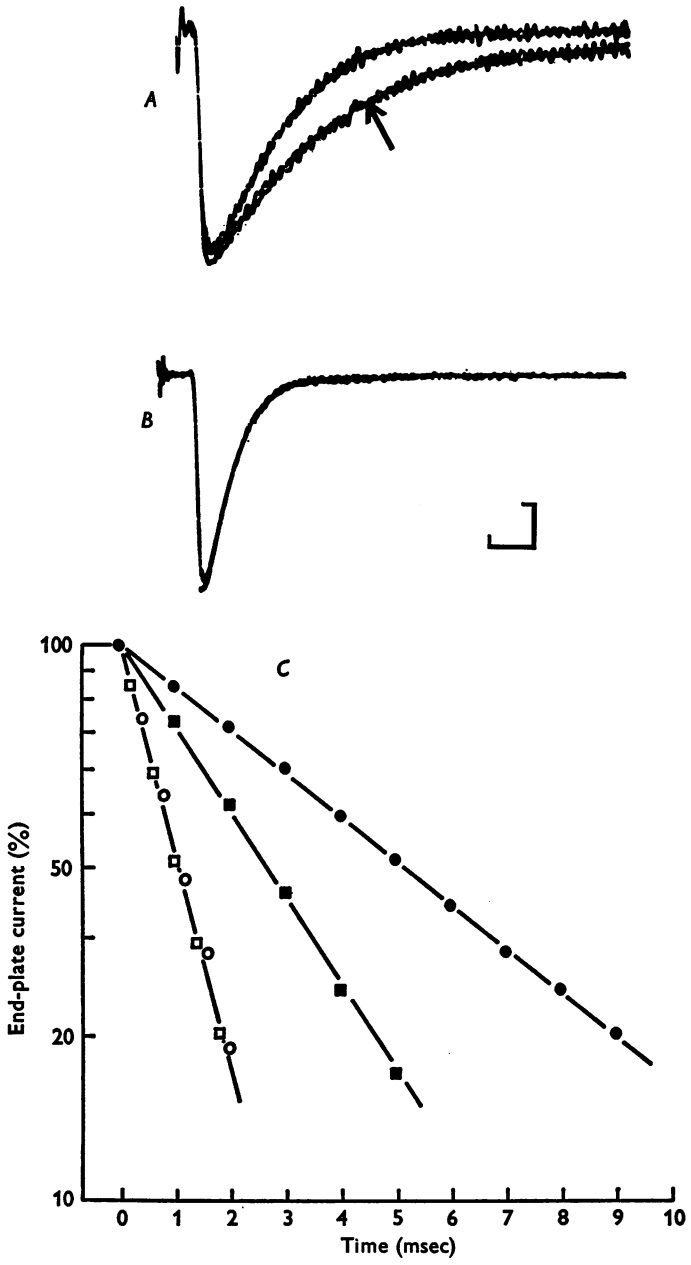

Fig. 9. *A*, *B* and *C*. For legend see facing page.

quickening of end-plate currents in prostigmine if desensitization inactivates receptors by changing their molecular conformation (Katz & Thesleff, 1957; Rang & Ritter, 1970; Miledi & Potter, 1971; Lester, 1972*b*). In this case our data suggest that desensitized receptors do not bind and/or release ACh with normal kinetics for if they did the factor *mt* (from the delayed diffusion hypothesis) and the resulting decay of end-plate currents would be unchanged. The data are not sufficient to indicate the contribution of possible decreased transmitter release to the quickening of end-plate currents which occurs during prolonged exposures to bath applied ACh or carbachol in the presence of prostigmine.

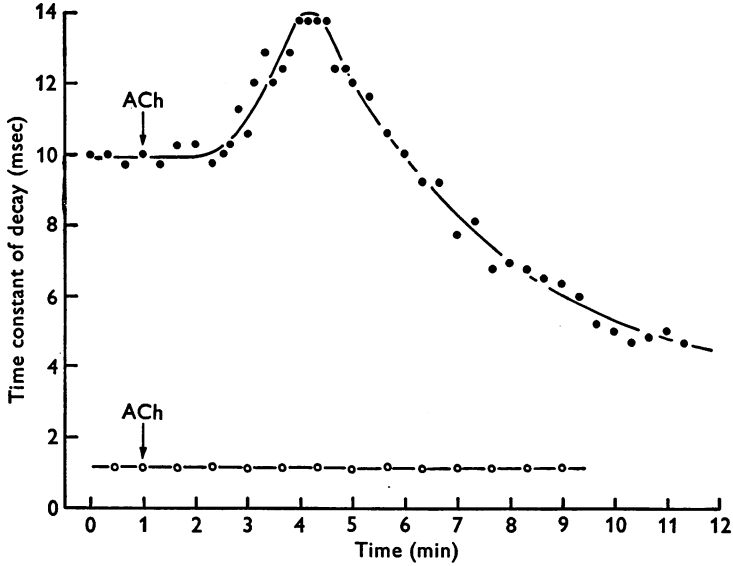

Fig. 10. Time constant of decay of end-plate current plotted as a function of the duration of exposure to bath applied ACh (20 μM). Filled circles: with prostigmine. Open circles: without prostigmine. The arrow indicates when the perfusion solution was switched to one containing ACh. Different preparations from Fig. 9. Holding potential: filled circles, -22 mV; open circles, -49 mV.

DISCUSSION

The data presented in the first part of this paper can be interpreted in terms of a single hypothesis: when acetylcholinesterase is blocked (by prostigmine, edrophonium, or collagenase treatment) repeated binding of ACh to receptors can prolong end-plate currents, the prolongation being less marked when the binding capacity of the post-synaptic membrane is reduced (with cobratoxin, α-bungarotoxin, or curare). Katz & Miledi (1973*a*) have suggested that repeated binding of ACh to receptors can

contribute to the prolongation of externally recorded miniature e.p.p.s in the presence of prostigmine. Our data provide further tests of this hypothesis extending it to end-plate currents. Our data do not exclude a series of hypotheses that each of the treatments described modify the mean lifetime of channels activated by ACh, prolongation being caused by prostigmine, edrophonium, and collagenase treatment, shortening being caused by cobratoxin, α-burgarotoxin, or curare. There are precedents for drug effects on channel lifetime, though this is usually seen at high concentrations of the modifying drug (Steinbach, 1968*a*, *b*; Katz & Miledi, 1973*c*; Kuba *et al.* 1973). However, Katz & Miledi (1971, 1972, 1973*a*, *b*) have presented evidence, based on a spectral analysis of voltage-noise accompanying the action of ACh, to suggest that the mean lifetime of conductance channels activated by ACh is not greatly affected by prostigmine, curare, or α-bungarotoxin. In view of these findings, it seems most reasonable to initially discuss our data in terms of the hypothesis that repeated binding of ACh to receptors can contribute to the prolongation of end-plate currents which occurs when acetylcholinesterase is blocked.

On the basis of this hypothesis, the prolongation of end-plate currents in the presence of prostigmine can be visualized in terms of individual ACh molecules repeatedly binding and activating receptors as the ACh molecules diffuse out of the synaptic cleft. If, on the average, each ACh molecule makes m successful bindings to receptors, each lasting t msec, then the time for diffusion of ACh from the synaptic cleft will be prolonged by mt msec leading to a prolongation of end-plate currents. Reducing the number of free receptors, for example with cobratoxin, α-bungarotoxin, or curare, should decrease the number of successful bindings of ACh to receptors as ACh diffuses from the synaptic cleft so that end-plate currents decay faster as we observed.

Our finding that the time constant of decay of end-plate currents in the presence of prostigmine increases with the concentration of ACh in the synaptic cleft seems not to be compatible with receptor models which assume that receptors are independent and do not interact. If receptors (defined as capable of binding a single ACh molecule) were independent and non-interacting the time required for an individual ACh molecule to diffuse from the synaptic cleft should not depend on the number of other ACh molecules in the synaptic cleft, provided that the number of free receptors is high. If a significant number of receptors were occupied, as might be the case in high concentrations of ACh, the time required for an individual ACh molecule to diffuse from the cleft should be decreased since the number of receptors available for an ACh molecule to bind to as it diffuses from the cleft is reduced. Our observation that end-plate currents decay slower in prostigmine when cleft [ACh] is raised, instead of faster as

suggested by the independent non-interacting receptor model, suggests that receptors are not independent but do interact. Our hypothesis for this *cooperative* action of ACh is that the association of ACh with one binding site favours the binding or retention of ACh at other sites. This cooperative action would arise if increasing the [ACh] in the synaptic cleft led to (1) an increase in m, the number of successful collisions of ACh molecules with receptors as they diffuse from the cleft, or (2) an increase in t, the time during which an ACh molecule is retained on a receptor. For example, an increase in m could result from an increase in the concentration of ACh in the synaptic cleft if two receptors in a functional unit had to be simultaneously occupied before ACh was retained for t msec. An increase in t could result from an increase in the concentration of ACh in the synaptic cleft if two occupied receptors in a functional unit retained ACh longer than a single occupied receptor.

More generally then the cooperative action of ACh on receptors can be represented as

$$n\mathrm{ACh} + n\mathrm{R} \rightleftharpoons \mathrm{ACh_n R_n} \rightleftharpoons \mathrm{ACh_n R_n^*}, \qquad (2)$$

where R represents the ACh receptor, $\mathrm{ACh_n R_n}$ is the ACh-receptor complex associated with a closed channel, and $\mathrm{ACh_n R_n^*}$ is the 'open' conformation of this complex, and n is an integer. The number n of ACh-molecules required to open a channel is not known but our data suggest that under some experimental conditions (Figs. 7, 8, 9, 10) n must be at least two for some of the ACh-receptor complexes. It is possible that ACh-receptor complexes may open a conductance channel with different numbers of ACh molecules (different values of n) and that each of these kinds of complexes may display different kinetics of binding of ACh to receptors and different kinetics of conformational changes associated with the opening and closing of conductance channels. If the ACh remains bound to the receptor when the conductance channel is open, an increase in channel lifetime would lead to an increase in t. Thus another possible explanation for the cooperative action of ACh on receptors is that at higher concentrations of ACh more ACh molecules combine with the receptor complexes so that these complexes adopt a conformation associated with a longer characteristic mean channel lifetime. Supporting this suggestion is the observation of V. Dionne & C. F. Stevens (personal communication) that in the presence of prostigmine, when ACh would be expected to persist in the region of receptors, the shape of the power spectrum of end-plate current fluctuations produced by ACh cannot be described assuming a single characteristic mean lifetime of conductance channels. In terms of the model described by equation (2) then, the present data cannot distinguish whether the cooperative action of ACh on receptors arises from

an increase in the number of bindings (m) or retention (t) of ACh to receptors as it escapes from the synaptic cleft. Prostigmine, edrophonium, and collagenase treatment would all be expected to increase the concentration of ACh in the synaptic cleft and consequently increase the chance for a co-operative action of ACh on receptors. If the cooperative action of ACh leads to a longer channel lifetime, then part of the prolonging effect of prostigmine, edrophonium, and collagenase on end-plate currents may be through an increase in channel lifetime as well as through multiple binding of ACh as it escapes from the synaptic cleft.

The proposed cooperative action of ACh on receptors provides a possible explanation for the observation in Fig. 6 that the time constant for the decay of end-plate currents in prostigmine did not decline linearly with the peak end-plate currents as α-burgarotoxin progressively blocked receptors. The toxin may block receptors leading to a direct decrease in the chance for a cooperative action of ACh on receptors or the toxin may act indirectly through its effect on the concentration of ACh in the synaptic cleft. Blocking receptors decreases the chance for multiple binding and hence decreases the concentration of ACh in the synaptic cleft. If binding or retention of ACh is cooperative, decreasing cleft [ACh] would lead to a greater than linear decrease in binding or retention of ACh and hence a greater than linear decrease in the time constant of decay of end-plate currents.

It is not immediately obvious to us in view of the demonstration of a cooperative action of ACh on receptors why end-plate currents should decay exponentially over most of their time course in the presence of prostigmine. In terms of the repeated binding and cooperative hypotheses it might be expected that end-plate currents would decay progressively faster with time and indeed this was observed in some experiments (see Fig. 8*A*) although in most experiments any deviation from an exponential decay was small.

It is of interest here that a cooperativity in the binding of ACh to membrane fragments from *Torpedo* electroplaques has been reported by Eldefrawi & Eldefrawi (1973) and by Weber & Changeux (1974). However, Moody, Schmidt & Raftery (1973) did not find a cooperativity in the binding of ACh to detergent-dispersed, receptor proteins purified from *Torpedo* electroplaques.

It should also be mentioned that dose–response curves (plotted linearly) for the action of ACh and other depolarizing compounds have been found to begin with a region of increasing slope both in the presence and absence of prostigmine. This has been observed at the frog neuromuscular junction (Katz & Thesleff, 1957; Jenkinson, 1960; Rang, 1971; Jenkinson & Terrar, 1973), and on isolated electroplaques from the electric eel (Higman,

Podleski & Bartels, 1963; Karlin, 1967; Changeux & Podleski, 1968). These observations have sometimes been interpreted in terms of a cooperative action of ACh on post-synaptic receptors (Karlin, 1967; Changeux & Podleski, 1968) though other interpretations are also possible (Rang, 1971; Jenkinson & Terrar, 1973).

In summary, our data suggest (1) that repeated binding of ACh molecules to receptors on their route of escape from the synaptic cleft can contribute to the prolongation of end-plate currents which occurs in the presence of prostigmine, and (2) that there can be a cooperative interaction between ACh and receptors. It should be mentioned once again that in the absence of prostigmine when ACh esterase is functional there is little or no repeated activation of receptors by ACh so that the time course of decay of end-plate currents is determined by the lifetimes of conductance channels (Magleby & Stevens, 1972*a*; Anderson & Stevens, 1973). The rapid hydrolysis of ACh by acetylcholinesterase and the possible cooperative activation of receptors by ACh could account for lack of repeated activation of receptors in the absence of prostigmine.

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