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AN ATTEMPT AT AN ANALYSIS OF THE FACTORS DETERMINING THE TIME COURSE OF THE END-PLATE CURRENT

I. THE EFFECTS OF PROSTIGMINE AND OF THE RATIO OF Mg^{2+} TO Ca^{2+}

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SUMMARY

1. An attempt was made to analyse the factors which might determine the time course of the falling phase of the end-plate current.

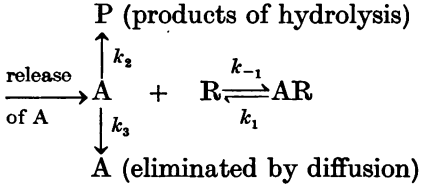
2. The end-plate current was measured by 'clamping' the membrane potential during neuromuscular transmission.

3. The falling phase of the end-plate current was found to consist of a non-exponential, and an exponential portion, the duration of both of which varies under different experimental conditions.

4. The results suggest that the rate constants determining the dissociation of the receptor–mediator complex seem greatly to influence the time course of the end-plate current.

INTRODUCTION

During neuromuscular transmission the released transmitter (A) is supposed to combine partly reversibly with receptors (R) in the end-plate membrane, and partly to be eliminated by hydrolysis and diffusion. In the light of work done hitherto on this problem, these processes can be summarized in the following kinetic scheme:



Upon the formation of the receptor–mediator complex (RA) the conductance of the junctional membrane is increased, giving rise to the end-plate potential (e.p.p.). By 'voltage-clamping' the membrane potential during neuromuscular transmission, the time course of the transmitter-induced conductance change can be studied by recording the end-plate current (e.p.c.; Takeuchi & Takeuchi, 1959).

The time course of the e.p.c. is usually given by the rise time of the rising phase and by the half-decline time of the falling phase. Both phases seem to be related to several factors (e.g. the spread of transmitter along the receptive surface, the junctional cholinesterase activity, the quantum content of the e.p.c., the degree of synchronization of the released quanta, cf. Takeuchi & Takeuchi, 1959; Beranek & Vyskočil, 1968; Kordaš, 1968*a*, *b*; see also Hubbard, Llinas & Quastel, 1969), but a more detailed analysis of these factors is lacking.

Although direct evidence is lacking, it will be assumed for simplicity (see e.g. Steinbach, 1968*a*, *b*; Werman, 1969) that the law of mass action can be applied to the reaction between A and R, and that the conductance increase (and so the e.p.c.) is linearly related to [AR]. It will be further assumed that the rate at which the conductance increase follows the formation of AR is very high. If this is so, the time course of [AR] is equal to the time course of the e.p.c. Applying the proposed kinetic scheme one would find that the time course of the e.p.c. is, in principle, dependent on the time course of the release of A, the concentration of A and of R and on k_1, k_{-1}, k_2 and k_3.

It is not possible to analyse the time course of the rising phase of the e.p.c. because the value of none of the factors mentioned above is known.

The falling phase of the e.p.c. seems to follow, as implied by the work of Maeno (1966), an exponential time course. This would mean that the concentration of AR is an exponential function of time. An analysis of the proposed kinetic scheme reveals (see Appendix) that this happens only if the time course of the release of A is relatively fast, k_1/k_{-1} relatively small and $k_1 \ll k_2+k_3$.

The aim of the present work was to find out whether these assumptions can be applied to the falling phase of the e.p.c. Thus an attempt was made (*a*) to establish whether the falling phase of the e.p.c. does follow an exponential time course and, if so, (*b*) to define the experimental conditions which might change it to a non-exponential time course. This could happen when k_2 is depressed, that is, when the hydrolysis of the transmitter is inhibited, as for example by prostigmine, or when the quantum content of the e.p.c. is high (cf. Kordaš, 1968*a*).

METHODS

All experiments were carried out at room temperature (18–22° C) on preparations of the sciatic nerve–sartorius muscle of the frog (*Rana esculenta*).

For the recording of the e.p.c. a modified voltage-clamp technique similar to that of Takeuchi & Takeuchi (1959) was used as described previously (Kordaš, 1968*b*).

The experiments were performed in two groups. In the first group the muscle was curarized by 3×10^{-6}–5×10^{-6} M tubocurarine (Nutritional Biochemical Corp. Cleveland; Mann Research Lab. New York) in Ringer solution of the following composition: NaCl 116 mM, KCl 2 mM, $CaCl_2$ 1·8 mM, Tris 4 mM, HCl 3·3 mM, pH 7·4. In a few experiments $CaCl_2$ concentration was increased to 3·6 mM in order to increase the e.p.c. Junctional cholinesterase was inhibited by prostigmine (S. A. F. Hoffmann, La Roche & Co. Basel) in the final concentration of 10^{-6}–10^{-5} M, for at least 20 min.

In the second group of experiments 'low-Na' Ringer was used: NaCl 23 mM, KCl 2 mM, $CaCl_2$ 3·6 mM, Tris 4 mM, HCl 3·3 mM, sucrose 168 mM, pH 7·4. Prostigmine was used in the final concentration from 10^{-6}–5×10^{-6} M. The quantum content of the e.p.c. was depressed by decreasing $CaCl_2$ to 0·3 mM, and by adding $MgCl_2$ to make up the final concentration of 3–5 mM.

In the latter group of experiments it was attempted to measure the quantum content of the e.p.c. This, however, was not feasible because nerve transmission failed soon after the muscle was immersed in the Na-deficient solution.

RESULTS

The time course of the falling phase of the e.p.c. in curarized muscle. Fig. 1 shows that immediately after the peak of the e.p.c. the time course is non-exponential, but only for about 0·5 msec or less. However, the rest of the falling phase follows an exponential time course with a half-time between 0·8 and 1·1 msec. If the junctional cholinesterase is inhibited by prostigmine, the non-exponential portion of the falling phase of the e.p.c. seems to last longer, and the exponential portion decays with the half-time between 1·4 and 1·7 msec (Fig. 2).

Since these data were obtained from different end-plates, the slower time course of the e.p.c. shown in Fig. 2 may not have been due specifically to prostigmine. Therefore, the e.p.c. was recorded in single end-plates before and after various amounts of prostigmine were added to the bath (Fig. 3). It is clear that after prostigmine-inhibition of junctional cholinesterase, the non-exponential portion of the falling phase of the e.p.c. lasts longer, whereas the exponential portion decays with a longer half-time. The latter finding is in good agreement with earlier results (Eccles, Katz & Kuffler, 1942; Eccles & MacFarlane, 1949; Takeuchi & Takeuchi, 1959; Kordaš, 1968*b*).

The transition of the e.p.c. from the rising phase to the falling phase is smooth. Thus it is clear that, since the major part of the falling phase is exponential, the initial portion of the falling phase must be non-

exponential. It is, however, less clear why following cholinesterase inhibition the duration of the non-exponential portion is increased. It is possible that this increase in duration is partly related to the spread of the trans-

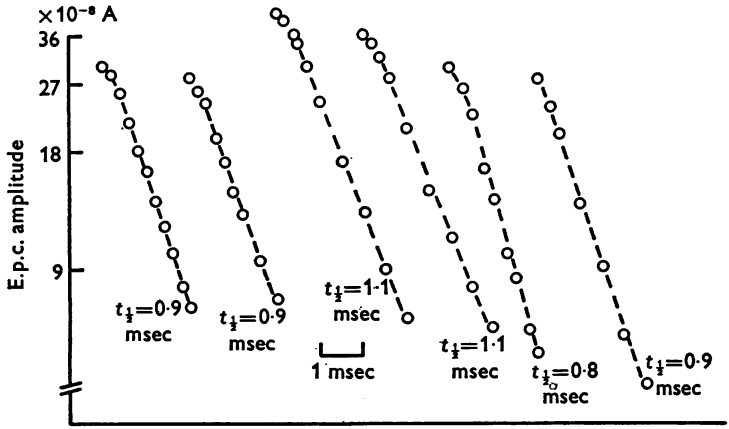


Fig. 1. The falling phase of the e.p.c. as recorded in six different end-plates in curarized muscle. In order to show the non-exponential and the exponential portion, the falling phase was plotted on a logarithmic amplitude scale.

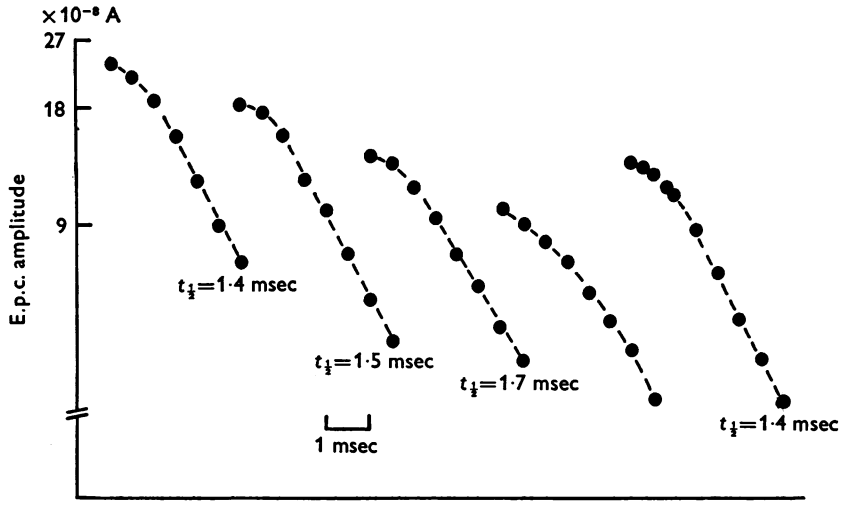


Fig. 2. The falling phase of the e.p.c. as recorded in five different end-plates in curarized muscle after treatment with 3×10^{-6} M prostigmine.

mitter along the receptive surface of the junctional membrane (cf. Eccles *et al.* 1942; Beranek & Vyskočil, 1968; Kordaš, 1968*b*). Therefore, a series of experiments was performed using a Na-deficient Ringer with twice

normal (i.e. 3·6 mM) Ca concentration. This experimental condition probably led to an increase in the quantum content of the e.p.c. (Jenkinson, 1957; Kelly, 1968; Birks & Cohen, 1965; Rahamimoff, 1968; Colomo & Rahamimoff, 1968); if in addition junctional cholinesterase is inhibited, a considerable spread of the transmitter along the receptive surface will occur.

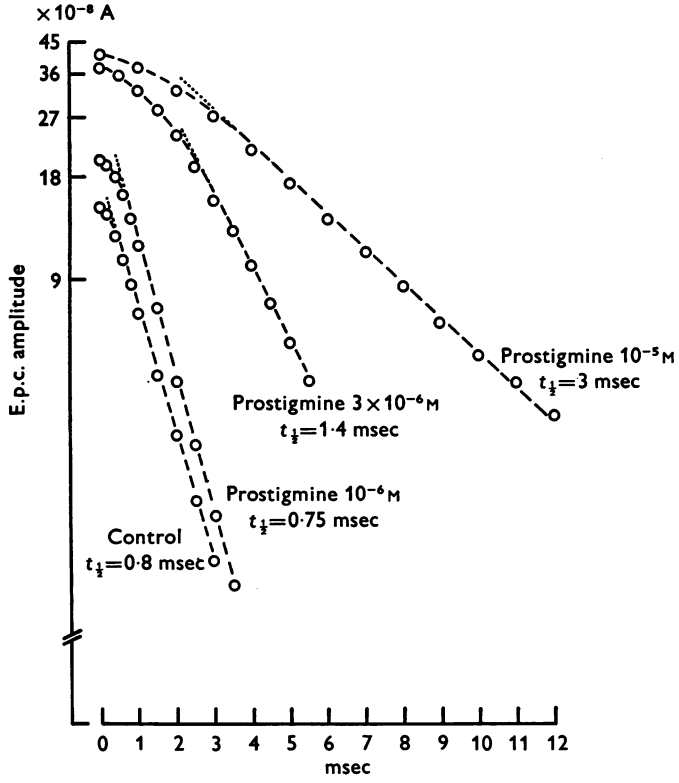


Fig. 3. The falling phase of the e.p.c. in curarized muscle before (control) and after treatment of the muscle with prostigmine in final concentration from 10^{-6} to 10^{-5} M. All data from the same end-plate.

The time course of the falling phase of the e.p.c. in muscle treated with low-Na Ringer. Under these experimental conditions the major part of the falling phase of the e.p.c. has an exponential time course with a half-time of about 1·4 msec. If prostigmine is added, the non-exponential portion becomes considerably longer (Fig. 4). These results confirm earlier findings (Fatt & Katz, 1951; Kordaš, 1968*a*) that anticholinesterases have a much stronger effect in muscle treated with a Na-deficient solution than they have in curarized muscles. It was suggested that this effect is

due to a pronounced spread of the transmitter along the receptive surface or, alternatively, to the saturation of receptors with the released transmitter (Kordaš, 1968*a*). If this is so, a reduction of the quantum content of the e.p.c. should have a striking effect on the time course. Therefore, in the experiments performed with low-Na Ringer with prostigmine, the quantum content of the e.p.c. was made either relatively high or relatively low.

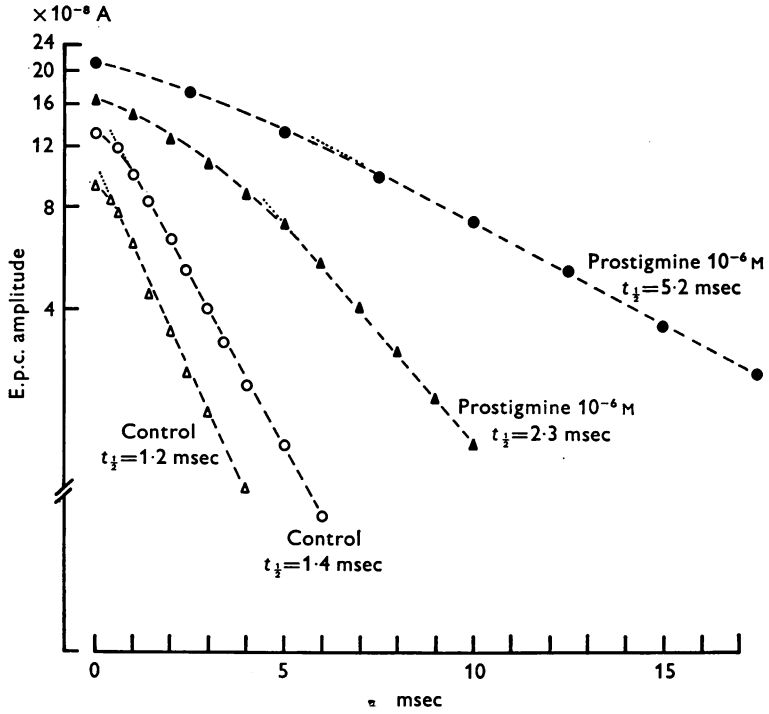


Fig. 4. The falling phase of the e.p.c. recorded in two different end-plates of muscle treated with Na-deficient Ringer solution before (open symbols) and after (filled symbols) treatment with 10^{-6} M prostigmine.

The effect of the quantum content of the released transmitter on the time course of the e.p.c. in muscles treated with Na-deficient Ringer and prostigmine. Under these experimental conditions the time course of the e.p.c. varies considerably in different end-plates. In some of them the e.p.c. has a 'flat top', whereas in others it is relatively 'pointed' (Fig. 5, cf. also Kordaš, 1968*a*, Figs. 2, 5 and 6). If the release of the mediator is further increased by applying two stimuli in rapid succession (Hutter, 1952; Eccles, 1952; del Castillo & Katz, 1954), the second e.p.c. is much slower and has a smaller amplitude than the first one (Fig. 5*A*). If, however, the

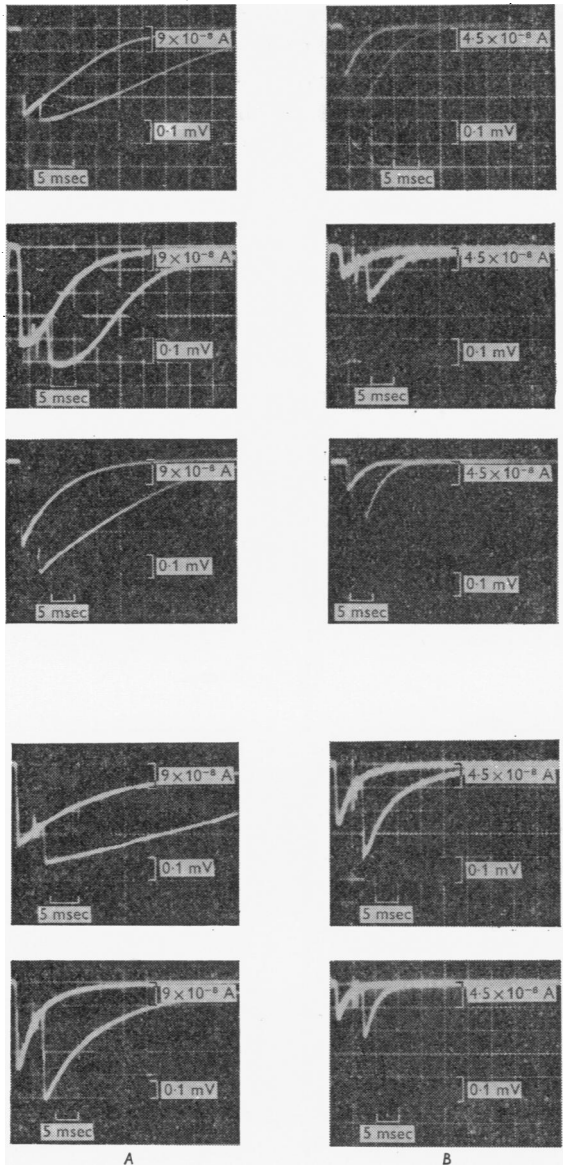


Fig. 5. Records of e.p.c. in five different end-plates of muscles treated with low-Na Ringer and 10^{-6} M prostigmine (upper three records) or low-Na Ringer and 5×10^{-6} M prostigmine (lower two records). For greater clarity, the lower traces (the intracellular recordings of the reduced membrane potential change during the flow of the 'clamping current') have been 'blacked out'. The e.p.c. is recorded on the upper beam (inward current downward). In all records the e.p.c.s were evoked by paired indirect stimuli. *A*: the quantum content of the e.p.c. is relatively high ($CaCl_2$ 3·6 mM). *B*: the quantum content of the e.p.c. is relatively low ($CaCl_2$ 0·3 mM; $MgCl_2$ 5 mM).

quantum content of the e.p.c. is depressed the time course of the two e.p.c.s becomes much faster, despite the presence of prostigmine (Fig. 5*B*). When the quantum content was relatively high, the time course of the falling phase of the one and of the other e.p.c. were greatly dissimilar.

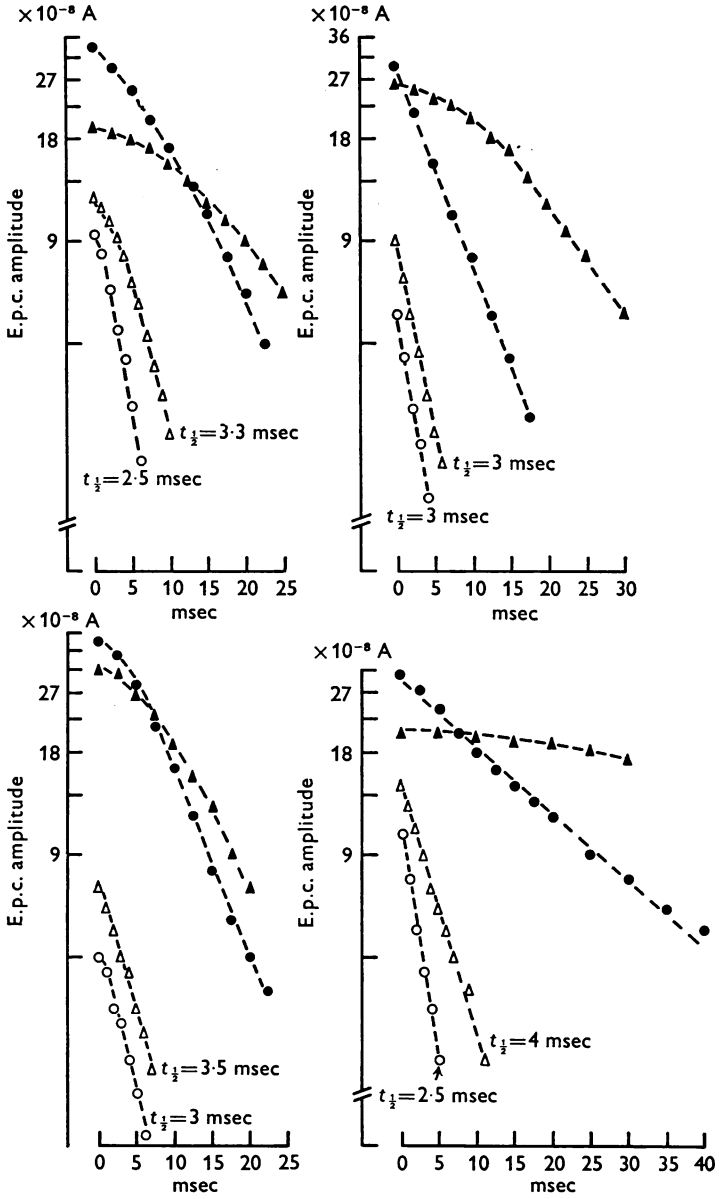


Fig. 6. For legend see foot of facing page.

On the other hand, when the quantum content was relatively low the falling phases of the two e.p.c.s were exponential and had similar half-times (Fig. 6).

DISCUSSION

The results presented here are in good agreement with earlier data (Eccles *et al.* 1942; Eccles & MacFarlane, 1949; Fatt & Katz, 1951; Takeuchi & Takeuchi, 1959; Kordaš, 1968*a*, *b*) and show that the time course of the e.p.c. depends both on the junctional cholinesterase activity and on the quantum content of the released transmitter (cf. Hubbard & Willis, 1962; Kuba & Tomita, 1971). The results further indicate that under some experimental conditions (either uninhibited junctional cholinesterase, or inhibited junctional cholinesterase and low quantum content of the released transmitter) the falling phase of the e.p.c. shows a very short non-exponential portion, while the rest of the e.p.c. decays exponentially.

On cholinesterase inhibition the non-exponential portion becomes longer while the exponential one has a longer half-time. If in addition to cholinesterase inhibition the quantum content of the e.p.c. is drastically increased, the non-exponential portion often becomes strikingly longer.

Since the transition of the rising phase of the e.p.c. into the falling phase is smooth, and since the major part of the latter is exponential, it is clear that the initial portion of the falling phase must be non-exponential. The length of the non-exponential portion, however, is different under different experimental conditions. Even though the reasons for this are not clear, it might be assumed that factors not incorporated in the proposed kinetic scheme are involved (e.g. a relatively asynchronous release of quanta, or the activation of receptors relatively distant from the point of quantal release; cf. Katz & Miledi, 1965; Kordaš, 1968*a*; Hubbard & Willis, 1962; Kuba & Tomita, 1971). Both the former and the latter mechanism may be operative when the quantum content is high, and the latter also on cholinesterase inhibition. The activation of distant receptors is probably also related to the geometry of the synaptic cleft;

Fig. 6. The effect of the quantum content of the e.p.c. on the falling phase of the e.p.c. in four different end-plates of muscle treated with low-Na Ringer and prostigmine. E.p.c.s were evoked by paired indirect stimuli (cf. Fig. 5). The falling phase of the e.p.c. evoked by the first (circles) and second (triangles) nerve stimulus. Filled symbols: quantum content relatively high ($CaCl_2$ 3·6 mM). Note that the second e.p.c. is slower and has a lower amplitude than the first one. Open symbols: quantum content relatively low ($CaCl_2$ 0·3 mM; $MgCl_2$ 5 mM). Note that the second e.p.c. has a similar time course and higher amplitude than the first one. The concentration of prostigmine was 5×10^{-6} M in the experiment shown in the lower right diagram, and 10^{-6} M in experiments shown in other diagrams.

therefore the time course of the e.p.c. can vary considerably in different end-plates when quantum content is high (cf. Figs. 5 and 6).

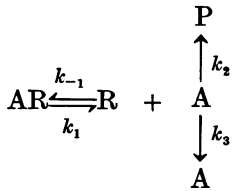
The exponential portion of the falling phase of the e.p.c. was analysed in the light of the proposed kinetic scheme (see Appendix). It seems that the exponential portion is due to the fact that k_1/k_{-1} is small and $k_1 \ll k_2 + k_3$. The effect of anticholinesterases can be explained by means of the same scheme, as k_2 is decreased by these drugs. Therefore the lengthening of the half-time of the exponential portion (see Appendix), affected by prostigmine, was expected. However, it is possible that also another mechanism is involved. Prostigmine in a relatively high concentration is known to depress the amplitude of the end-plate potential (Eccles & MacFarlane, 1949), i.e. to have curare-like properties. In the strictest sense of this term this would mean that this drug, like curare, combines with receptors, the resulting complex being inactivable by the released A, and the net result being a reduction of the number of receptors free for combination with A. In a broader sense of this term, however, it can be speculated that the prostigmine-receptor complex is still, but only to a small extent, activable by the released A. In this case it would be possible that also k_1 is decreased, or k_{-1} increased, whereby the half-time of the exponential portion is lengthened correspondingly.

In the light of the present, mostly descriptive data it is suggested that not only the hydrolysis of the mediator (k_2) and its diffusion out of the synaptic cleft (k_3), but also the value of k_1/k_{-1} is important for the determination of the time course of the e.p.c. Experiments which provide further data on this problem will be presented in a separate paper (Kordaš, 1972).

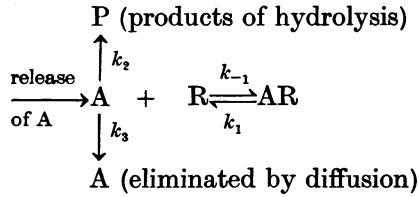
APPENDIX

By I. Gabrovec, M. Kordaš and B. Popovič

It was felt that the proposed kinetic scheme should be analysed more accurately in order to find those values of rate constants which would make the AR concentration an exponential function of time. For this reason a kinetic analysis was made for two different sets of initial conditions:



First set of initial conditions



Second set of initial conditions

An analogue computer programme was set up (Fig. 7*A*) for the calculation of [AR], [A], and [R] for the two different sets of initial conditions. It is assumed that in the first set R is attached to the walls of a small

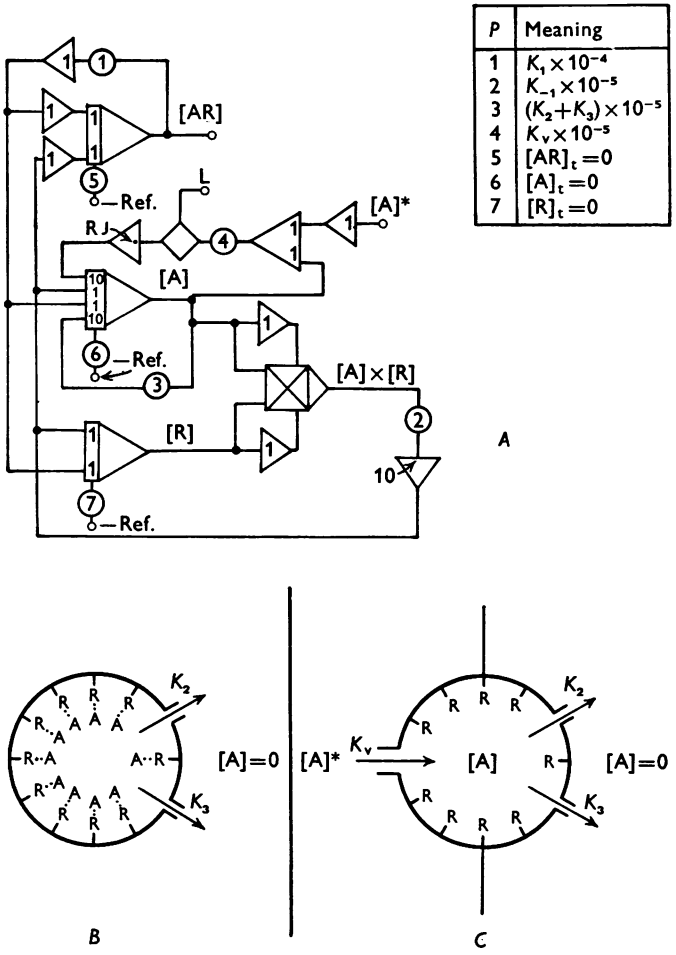


Fig. 7 *A*. The analogue computer programme for the calculation of [AR] and [A] for the two different sets of initial conditions; the logical signal was thus either low (L = '0') or high (L = '1'). [A]* was simulated by a square pulse. The two sets of initial conditions are graphically shown in (*B*) and (*C*).

vessel, and A reversibly attached to R. At the beginning of the reaction only AR is present. Because outside of the vessel [A] = 0, A is eliminated from the vessel via two channels, representing k_2 and k_3. It is further assumed that the vessel is so small that inside it there are no concentration gradients of A (Fig. 7 *B*).

The system is described by the following equations:

$$\frac{d[AR]}{dt} = -k_{-1}[AR] + k_{-1}[A].[R], \tag{1}$$

$$\frac{d[A]}{dt} = k_1[AR] - k_{-1}[A].[R] - (k_2 + k_3).[A], \tag{2}$$

$$\frac{d[R]}{dt} = -\frac{d[AR]}{dt} = k_1[AR] - k_{-1}[A].[R]. \tag{3}$$

Eq. (1) shows that if $k_{-1}.[A].[R]$ is small in comparison with $k_1.[AR]$, [AR] will be an exponential function of time.

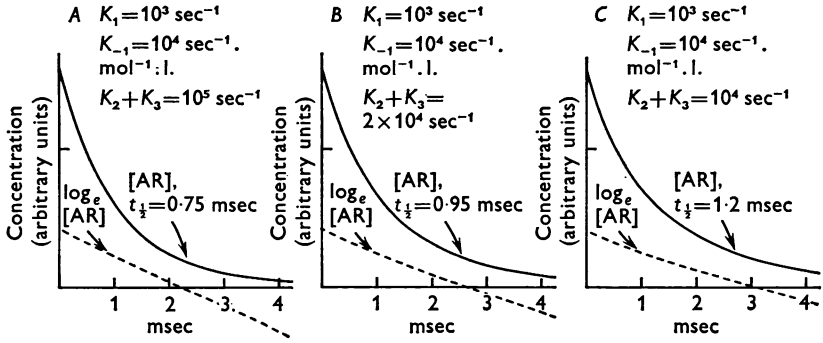


Fig. 8. Initial conditions as shown in Fig. 7*B*. The time course of [AR] when k_1 and k_{-1} were kept constant and the value of k_2+k_3 was varied. For checking the exponential decay of [AR], log$_e$ [AR] is shown in each graph by a dashed line. Note that in *A*, where the value of k_2+k_3 is relatively high, the latter is straight, whereas in *B* and *C*, where the value of k_2+k_3 is relatively low, it is slightly bent at the beginning of the reaction.

For the sake of simplicity anticholinesterase action is simulated by decreasing the value of k_2+k_3, although it is clear that only the former constant is affected by these drugs. Further, the effect of the 'stability' of AR is simulated by varying k_1 and k_{-1}.

It can be seen that, provided k_1/k_{-1} is small and $k_1 \ll k_2+k_3$, [AR] is an exponential function of time. This is compatible with the view that the proposed kinetic scheme illustrates mechanisms, underlying the so called exponential portion of the falling phase of the e.p.c. As shown in Fig. 8, the time course of [AR] is almost strictly exponential when $k_1 = (k_2+k_3)/100$. When, however, $k_1 = (k_2+k_3)/20$ or $k_1 = (k_2+k_3)/10$, both of which simulate anticholinesterase action, the time course, at the beginning of the reaction is no longer strictly exponential, and the half-time of [AR] decay is longer (Fig. 8*B*, *C*). While the deviation in the time course is too small to be detected in the falling phase of the e.p.c.,

the lengthening of the half-time of [AR] is pronounced enough to lengthen the decay of the e.p.c. Further, an increase in k_{-1} or decrease in k_1 results in a striking lengthening of the [AR] decay (Fig. 9).

It is clear that this set of initial conditions is a gross simplification of the processes involved in neuromuscular transmission. Therefore, the proposed kinetic scheme is analysed for another, better set of initial conditions.

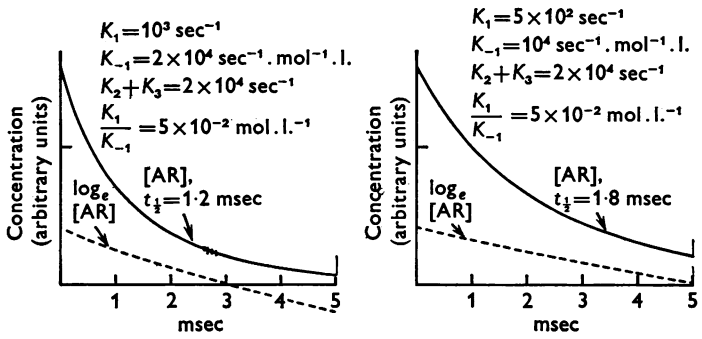


Fig. 9. Initial conditions as shown in Fig. 7*B*. The time course of [AR] when either k_1 or k_{-1} are varied, while keeping $k_1/k_{-1} = 5\times10^{-2}$ M, the latter being twice smaller from the value stated in Fig. 8*B*. Note the striking lengthening of time course of [AR], the roughly exponential decay of which being shown by $\log_e$[AR] by the dashed line.

In this second set of initial conditions it is assumed that R is again attached to the walls of the vessel, while A diffuses at a very high rate into the vessel from an outside source kept at a constant concentration ([A]*). The port allowing this diffusion is characterized by the constant k_v and by ([A]*−[A]), and is opened only for a fraction of a millisecond. On being released into the vessel, A enters into a reversible combination with R, and resulting AR dissociates because of the continuous removal of A, characterized by the rate constants k_2 and k_3 (Fig. 7*C*). By this set of initial conditions, both the rising and the falling phases of the e.p.c. can be simulated. In this case eq. (2) is modified to

$$\frac{d[A]}{dt} = k_v([A]^*-[A]) + k_1[AR] - k_{-1}[A][R] - (k_2+k_3)[A]. \qquad (2a)$$

If by means of an analogue computer (Fig. 7*A*) the values of [AR] and [A] are calculated from eqns. (1), (2*a*) and (3), interesting information is obtained (Fig. 10). If the time course of the release of A into the vessel, simulated by the square pulse [A]*, is kept constant, both [AR] and [A] are a function of k_2+k_3.

The decay of [AR] is exponential, while its half-time increases when the

value of k_2+k_3 decreases. In the initial portion of the falling phase of the [AR] decay there is a slight deviation from the exponential time course if the value of k_2+k_3 approaches that of k_1.

These considerations support the view that the mechanisms underlying the exponential portion of the falling phase of the e.p.c. can be explained

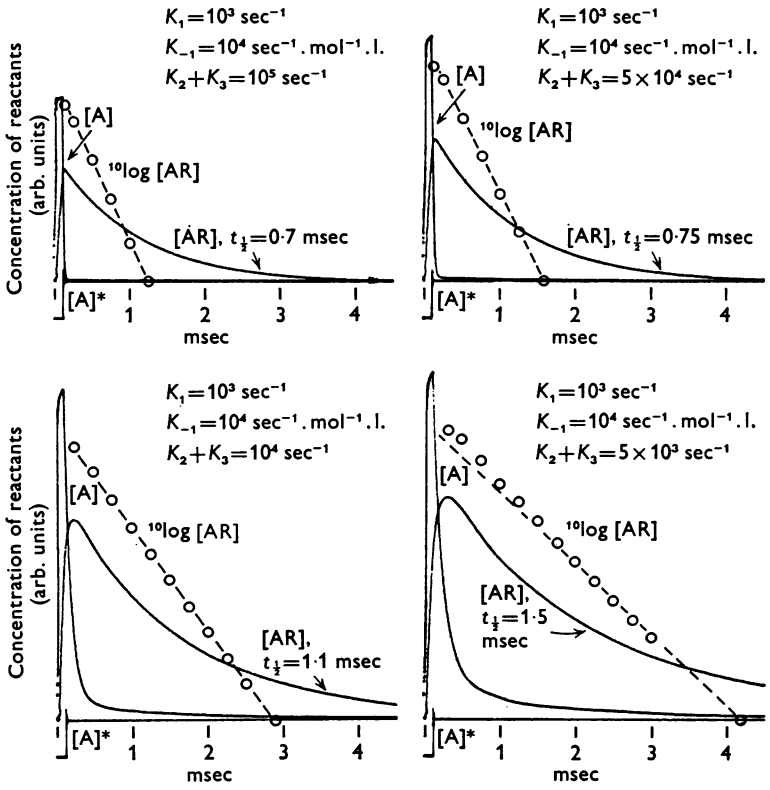


Fig. 10. Initial conditions as shown in Fig. 7*C*. The time course of [AR] and [A] when the release of A ([A]*) is constant, while the value of k_2+k_3 is varied. To check the exponential decay of [AR], ^{10}log [AR] is shown in each graph by circles. Note that, when the value of k_2+k_3 is decreased, there is a slight deviation from exponential time course, and a lengthening of half-time of the falling phase of [AR].

in the light of the proposed kinetic scheme. On the other hand, the simulation experiments do not afford a satisfactory explanation of the rising phase and the non-exponential portion of the falling phase of the e.p.c. To simulate these, a better formulation of the diffusion of A and of the activation of the receptors in the synaptic cleft is needed.

Note added in proof

Data very similar to those described above have been obtained independently by K. L. Magleby & C. F. Stevens (*J. Physiol.* **223**, 151–171).

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