Interaction at end-plate receptors between different choline derivatives

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Interaction between different choline derivatives has been studied by applying them simultaneously to a motor end-plate and recording the resulting changes in the membrane potential of the muscle fibre.

Choline potentiates the depolarizing effect of acetylcholine (*Ach*) when applied in normal Ringer. Decamethonium has a 'diphasic' action, initial depression of the *Ach* effect being followed by more prolonged potentiation.

When these experiments are made after treating the muscle with an esterase inhibitor (prostigmine 10^{-6} w/v), the potentiation of the *Ach* effect, by decamethonium or choline, is absent and replaced by simple 'curare-like' inhibition.

When decamethonium is allowed to interact with a rapidly acting stable ester (carbaminoylcholine or succinylcholine), it produces simple 'curare-like' inhibition.

The triple effects of choline and decamethonium, i.e. (i) weak depolarization, (ii) potentiation of *Ach* in normal Ringer solution, (iii) inhibition of *Ach* in the presence of prostigmine, can be explained by competitive reactions between the drugs and receptor as well as *Ach*-esterase molecules. It is suggested that the first step in a depolarizing end-plate reaction is the formation of an intermediate, inactive, compound between drug and receptor.

Introduction

It is generally believed that there are two kinds of 'acetylcholine receptors' at the motor end-plate: (*a*) those whose reaction leads to depolarization (i.e. the 'receptor' in its usual sense) and (*b*) those which cause acetylcholine to become rapidly hydrolyzed (the histochemically identified cholinesterase). The two types can be differentiated by their relative specificity towards chemical inhibitors (e.g. curarine and prostigmine), though they may exist as different side groups of the same protein molecules (Župančič 1952). As regards the activity of cholinesterase, it has been shown to follow the Michaelis theory (Augustinsson 1948, 1950), according to which the enzyme reaction proceeds in two steps, the first being the formation of an intermediate compound between substrate S and enzyme E:

$$S+E \rightleftharpoons SE \rightarrow S_1+S_2+E. \tag{1}$$

We will suppose, as a working hypothesis, that the 'receptor' (in its restricted sense) reacts by a similar two-step process, first forming an intermediate inactive compound which is then changed into an active, depolarizing, form (cf. Kirschner & Stone 1951; Ariëns 1954). We may represent this, for instance, by

$$S+R \rightleftharpoons SR \rightleftharpoons SR', \tag{2}$$

where SR' is the depolarizing compound whose nature and transformation are, at present, unknown.

According to this concept, whether a substrate acts as a depolarizer or a competitive inhibitor would depend solely on the rate constants of the two steps;

d-tubocurarine, for instance, may be considered to form a reversible intermediate compound *SR* without proceeding to the next step. Moreover, a substance which by itself has relatively weak or slow depolarizing action (e.g. decamethonium, choline; cf. Castillo & Katz 1957*b*; and p. 374 below) may, at the same time, antagonize the depolarization produced by fast and powerful agents like acetylcholine and carbachol.

In general, if two depolarizing substances, e.g. acetylcholine (*Ach*) and a stable cholinester, are allowed to act simultaneously on a motor end-plate, various interactions may be foreseen from equations (1) and (2); most stable cholinesters are competitive inhibitors of the esterase and may, therefore, be expected to potentiate the *Ach* potential; at the same time, they combine with the receptor *R*, and if the second step of their reaction is slow, the *Ach* potential will be inhibited. The net result depends, therefore, on a balance of two opposite effects and on the relative affinities and rate constants involved in the two processes.

In the present experiments the method of rapid ionophoresis has been used to apply two depolarizing drugs simultaneously from a twin pipette and to study their interaction on end-plate receptors. A number of results will be described whose apparent complexity can be reduced to simple terms on the basis of the initial hypothesis, namely, that the first step in the drug/receptor action is the formation of an inert compound.

Methods

The method is identical with that described in the previous papers (Castillo & Katz 1957*a*, *b*), except that the ionophoretic pulses through the two barrels were timed simultaneously or in rapid succession so that the drugs were allowed to interact. In most experiments, twin micropipettes were used containing different combinations of the following drugs: acetylcholine (*Ach*), decamethonium (C_{10}), succinylcholine (*Succ*), carbaminoylcholine (carbachol, *Carb*), choline. Some experiments were also made with two separate drug pipettes to ascertain that the results obtained with 'twin pipettes' had not been vitiated by possible electrical interaction between the barrels. Of the substances used in this study, all except *Ach* may be regarded as 'stable', i.e. not subject to enzymic hydrolysis at the myoneural junction. Succinylcholine is known to be inactivated fairly rapidly *in vivo*, but according to Fraser (1954) the enzymic hydrolysis of this substance *in vitro* is slow compared with that of *Ach*, and almost entirely due to serum-esterase, while the 'specific' tissue enzyme appears to have little effect on succinylcholine.

Nomenclature

The terms 'inhibition' and 'potentiation' will be used to describe the two main types of drug interaction which were observed. By 'inhibition' we mean a reduction in the depolarizing effect of one drug due to addition of another drug. When both substances have a depolarizing effect, the term 'inhibition' is used only if the response to their combined application is *less* than the response to the more potent substance alone. The term 'potentiation' is used when the application of one drug increases the depolarizing effect of another drug.

Results

Interaction between acetylcholine and decamethonium

It was shown previously (Castillo & Katz 1957*b*) that a brief pulse of decamethonium (C_{10}) produces a much slower and weaker depolarization than a similar pulse of acetylcholine (*Ach*) or carbachol (*Carb*). This might conceivably have been attributed to a differential diffusion barrier making the receptors more readily

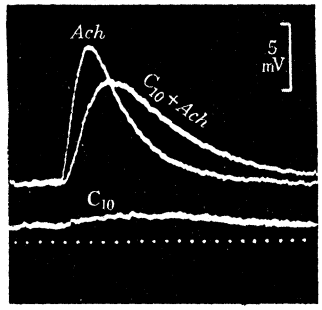

FIGURE 1. Interaction between C_{10} and *Ach*. Depolarizations produced by the two drugs separately (*Ach*, C_{10}) and combined ($C_{10}+Ach$). In the upper two records, a brief *Ach* pulse (13 ms, $4{\cdot}4\times10^{-10}$ C) was applied, first by itself, then immediately preceded by a longer C_{10} pulse (84 ms, $1{\cdot}7\times10^{-9}$ C). Time marks 50 c/s.

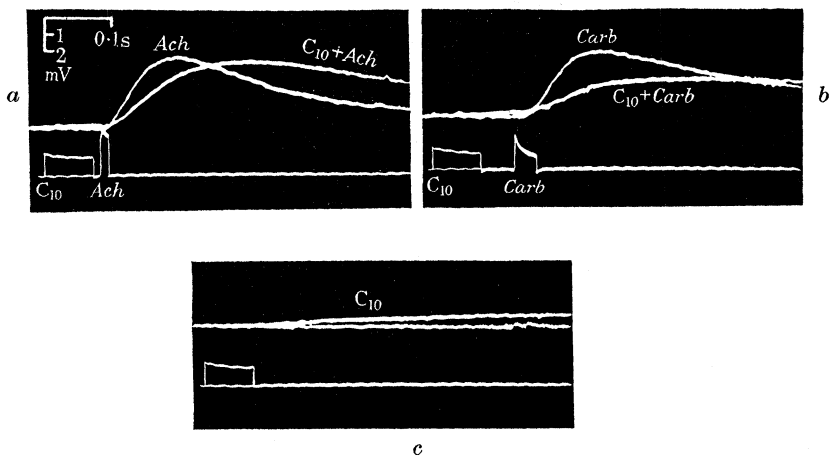

FIGURE 2. C_{10}/Ach (*a*) and $C_{10}/Carb$ (*b*) interactions. In each record, upper traces show membrane potential changes, lower traces the applied drug pulses. Monitor calibration: 1 scale div. = $1{\cdot}4\times18^{-8}$ A.

accessible to *Ach* and *Carb* than to C_{10}. Such an explanation, however, is contrary to the results shown in figures 1 and 2. When C_{10} and *Ach* are given together, the two potential changes do not add, but a rather complicated interaction occurs between the drug effects. In figure 1, for instance, the first effect of C_{10} is to reduce the rate of rise of an added *Ach* potential, at a time when the same dose of C_{10},

given by itself, produced a barely significant potential change. It appears, therefore, that the drug does, in fact, reach the receptors and react with them, even when no appreciable depolarization is observed. The whole time course of the *Ach* potential is modified and slowed in the presence of C_{10}, and during its decay the size of the potential considerably exceeds the sum of the two separate deflexions.

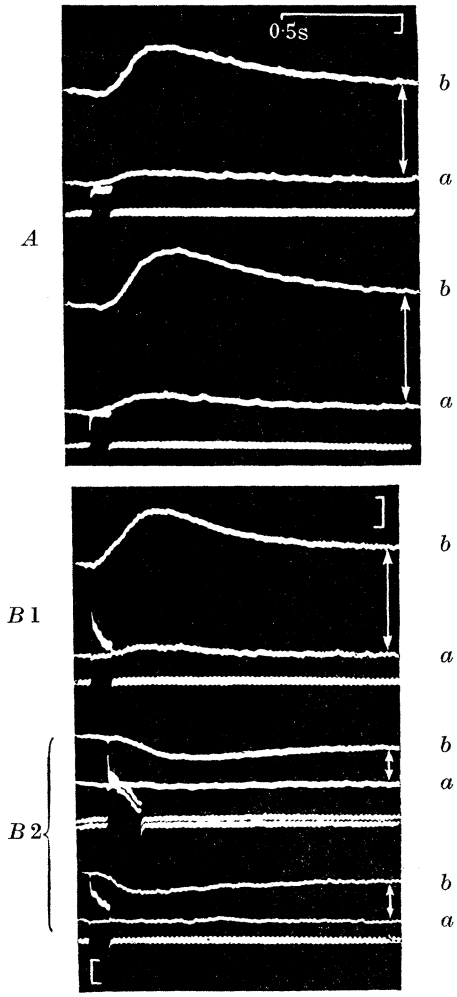

FIGURE 3. Effect of C_{10} pulses during steady *Ach* potentials. In each record, a C_{10} pulse was applied (*a*) in the absence of '*Ach* background', (*b*) when a steady *Ach* depolarization had been produced (by lowering the 'braking' current in the *Ach* barrel). *A*, from two end-plates in normal Ringer solution. *B*, from a single end-plate (1) before, (2) after prostigmine application. Vertical scales: Potential calibrations: 2 mV. Monitor calibrations: *A*, 9×10^{-9} A; *B* 1, $1{\cdot}6 \times 10^{-8}$ A; *B* 2, $1{\cdot}2 \times 10^{-8}$ A.

The exact form of the C_{10}/*Ach* interaction depended on a number of variables, such as the strength of the C_{10} pulse, its timing and the position of the pipette. We always observed a lengthening of the *Ach* potential, but the depression of its rate of rise was more variable and sometimes very slight. In these cases, the main effect of C_{10} was to produce an increase and lengthening of the *Ach* potential.

Another way of studying the interaction of these two substances was to depolarize the end-plate by a small steady efflux of *Ach* and superimpose a C_{10} pulse. Experiments of this kind are illustrated in figures 3, 4 and 7. The usual effect was a transient increase of the depolarization, corresponding presumably to the delayed potentiation of the pulse potential. In some cases, however (e.g. figure 4*a*), a diphasic effect was found, viz. an early depression followed by augmentation.

We shall try to interpret these complex features on the basis of our initial hypothesis, and then examine the validity of this interpretation by further experiments.

Suppose the reaction between decamethonium and the receptors proceeds in two steps of the following kind

$$C_{10} + R \rightleftharpoons RC_{10} \rightleftharpoons R'C_{10},$$

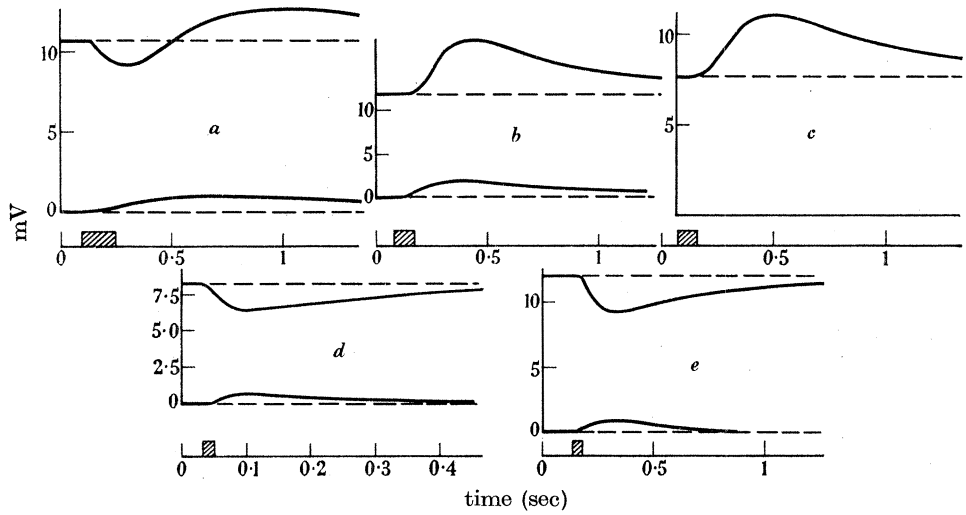

FIGURE 4. Effect of C_{10} pulses on steady *Ach* potentials. *Ach* potentials (indicated by the level of the upper tracings) were obtained by reducing the 'braking' current in the *Ach* barrel. Lower tracings show effect of C_{10} pulse by itself (it depolarized in all cases except (*c*)). Timing of C_{10} pulses shown by shaded rectangles. *a* to *c*, in normal Ringer solution. *d*, in prostigmine-Ringer solution (10^{-6} w/v). *e*, carbachol was used, instead of *Ach*, as a depolarizing agent. The potentiation of the *Ach* potential, in *a* to *c*, is absent in *d* and *e* and replaced by simple inhibition. Strength of C_{10} pulses ($\times 10^{-9}$ C): *a*, 2·4; *b*, 1·4; *c*, 1·2; *d*, 0·26; *e*, 0·9.

where the first step is the formation of an intermediate, inactive compound, and the second step its tranformation into a 'depolarizing' receptor complex. If the forward rate of the second reaction is slow, and that of the first reaction fast, then it would be easy to understand why the drug has a weak depolarizing effect and, at the same time, inhibits the formation of an *Ach* receptor compound. Furthermore, there is evidence that C_{10}, like many other stable cholinesters, forms an intermediate compound with, and therefore acts as a competitive inhibitor of, *Ach*-esterase (see Paton & Zaimis 1949; Goldstein 1951; Thesleff 1955; Discussion below). This would explain the prolongation, and 'delayed potentiation', of the *Ach* potential.

Our hypothesis has two consequences which can be tested experimentally. First, if the preparation is treated initially with an anti-esterase (e.g. prostigmine), then

the potentiating action of C_{10} should be reduced or eliminated, and its inhibitory effect alone would remain. Secondly, if C_{10} is allowed to interact with a fast, but stable, depolarizing agent (e.g. carbachol), the result should be a simple competitive inhibition, without delayed potentiation.

Effect of prostigmine on C_{10}/Ach *interaction*

When the experiments were repeated on muscles treated with prostigmine (10^{-6} w/v, > 30 min soaking), the interaction between C_{10} and *Ach* took on a much simpler appearance: C_{10} now simply depresses the *Ach* potential, whether this is set

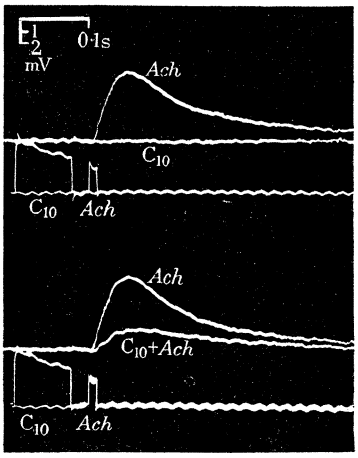

FIGURE 5

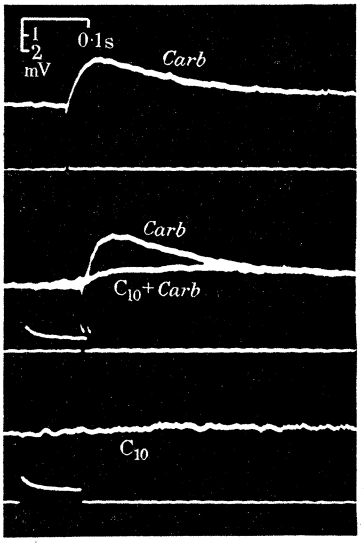

FIGURE 6

FIGURE 5. C_{10}/*Ach* interaction in prostigmine-treated muscle. The upper part shows two superimposed records, with *separate* application of *Ach* and C_{10} (C_{10} in this case producing no potential change by itself). The lower part shows the inhibitory action of C_{10}. Monitor calibration: 1 scale div. = 6×10^{-9} A.

FIGURE 6. Interaction between C_{10} and carbachol. Monitor calibration: 1 scale div. = $2{\cdot}3 \times 10^{-8}$ A.

up by brief pulses (figure 5) or by steady efflux (figures 3 and 4) from the *Ach* pipette. This 'curare-like' action was often observed even when the C_{10} pulses produced no noticeable effect by themselves.

This observation made us wonder whether the weak C_{10} depolarization observed in normal Ringer solution was not really an indirect effect, arising from esterase inhibition and allowing traces of *Ach* to become effective. It is difficult to eliminate this possibility, but there is, of course, no doubt that C_{10} can produce a large end-plate depolarization if applied in the more conventional way (e.g. Burns & Paton 1951; Thesleff 1955), and furthermore, we have seen C_{10} potentials in some at least of the prostigmine-treated end-plates.

The first prediction of our hypothesis, therefore, has been verified, namely, that in prostigmine-treated muscle, the complex interaction effects between C_{10} and *Ach* would be transformed into a simple 'curare-like' inhibition.

Interaction between C_{10} and stable esters

If instead of *Ach*, a stable ester like carbachol or succinylcholine is used in the second barrel of the pipette, the effect of C_{10} is again a simple inhibition of the drug potential. This is illustrated in figure 2, where the C_{10}/*Carb* interaction is compared with that of C_{10}/*Ach* at the same end-plate region, and further examples with pulsatile and steady applications of carbachol and succinylcholine are shown in figures 6, 7 and 8. Thus, the *potentiating* effect of C_{10} appears to be a special case, observed

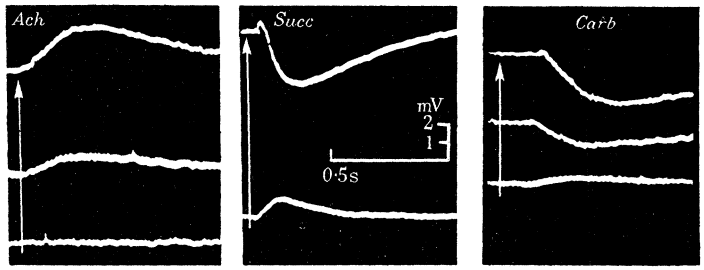

FIGURE 7. Effect of C_{10} pulses on steady drug potentials. The steady depolarizations (in the direction of the arrows) were produced, by gradual reduction of the 'braking current', with *Ach*, *Succ* and *Carb*, as indicated. A C_{10} pulse was applied at the beginning of each trace. C_{10} pulse strength and duration: *Ach*, $0{\cdot}75 \times 10^{-9}$ C, 75 ms.; *Succ*, $1{\cdot}1 \times 10^{-9}$ C, 50 ms; *Carb*, $2{\cdot}3 \times 10^{-9}$ C, 140 ms.

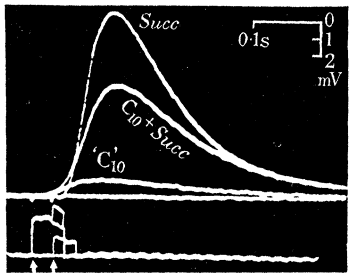

FIGURE 8. Interaction between C_{10} and succinylcholine. Three superimposed records showing inhibition of succinylcholine potential (produced by delayed small pulse) by a preceding C_{10} pulse. Arrows show beginning of C_{10} and *Succ* pulses. Monitor calibration: 1 scale div. = $8{\cdot}3 \times 10^{-9}$ A. (The small 'C_{10} potential' had an unusually fast time course; it is uncertain whether this was genuine or due to a small leakage of current between the barrels.)

only when it interacts with *Ach* in the absence of a potent cholinesterase inhibitor. In all the other cases, a 'curare-like' inhibition is the predominant or sole effect (see also Ariëns & de Groot 1954; Ariëns, Simonis & de Groot 1955).

Interaction between choline and acetylcholine

Choline was of interest, because this substance is produced locally in the course of normal neuro-muscular transmission. Choline has a very weak depolarizing action of its own (cf. Welsh & Taub 1948) and is known to be a competitive inhibitor of the esterase (Augustinsson 1948).

(*a*) *Choline potentials*

When choline was applied ionophoretically, its depolarizing power was found to be over a hundred times less than that of *Ach*, while the time course of decay of the potential was only slightly slower (e.g. figure 10). The following summarizes the results of ten experiments (five of them with prostigmine) in which *Ach* and choline were applied from twin pipettes. *Ach* pulses of $6{\cdot}2 \times 10^{-11}$ C (5 to 14 ms duration) produced a mean depolarization of 5·3 mV, with a half-decay time of 51 ms. Choline pulses of $1{\cdot}9 \times 10^{-9}$ C (50 to 140 ms) gave 0·57 mV depolarization with a half-decay occurring in approximately 90 msec.

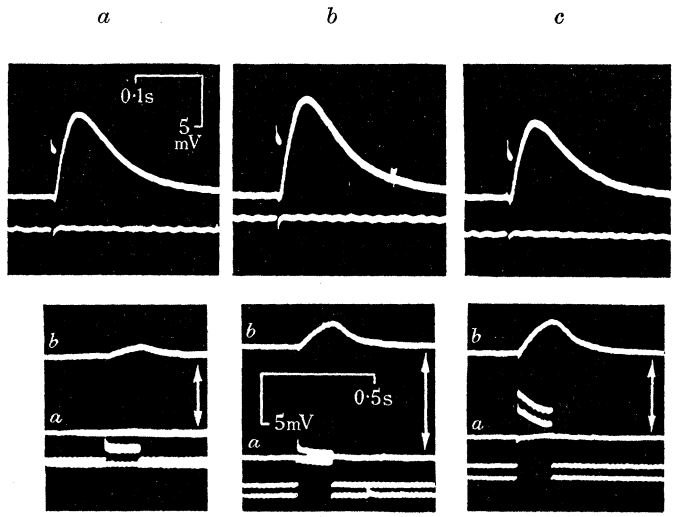

FIGURE 9. Potentiation of *Ach* potentials by choline. Upper part: *Ach* pulses applied normally (*a* and *c*), and during a steady efflux of choline from the other barrel (*b*). Lower part: Choline pulses applied normally (*a*) and during steady efflux of *Ach* from the other barrel (*b*). Monitor calibration ('5 mV' scale) = $2{\cdot}7 \times 10^{-8}$ A.

(*b*) *Interaction effects*

(i) *Potentiation.* When choline was applied during, or immediately preceding, the release of *Ach* from the other part of the pipette, a small but consistent interaction was observed. In normal Ringer solution, the *Ach* effect was potentiated (figures 9 and 11) by pulses of choline, even when these produced a hardly noticeable depolarization themselves. The potentiation was best shown when a choline pulse was superimposed on a steady efflux of *Ach*, as illustrated in figure 9 (lower part). The 'base-line' in these records shows that the choline pulse produced no significant potential change when the efflux of *Ach* had been stopped by the usual small 'braking' current (see Castillo & Katz 1957*a*, Methods). When this 'bias' was reduced, and a steady *Ach* potential had developed, the same choline pulse caused a transient increase of the depolarization. On the average, a choline discharge of $2{\cdot}3 \times 10^{-9}$ C increased an *Ach* depolarization of 8 mV by about 30 %. The half-time of decay of this potentiation occurred in about 80 to 90 ms (comparable to the half-time of the small 'choline potential' previously described).

(ii) *Inhibition.* When the same experiment was repeated after treating the muscle with prostigmine (10^{-6} w/v), the potentiation was reduced or abolished, and usually a simple inhibitory effect appeared, like the one observed under these conditions with C_{10}. Figure 10 shows an example: the choline pulse which, by itself, retains a weak depolarizing action, reduces the amplitude of an added *Ach* potential. In figure 11 the reversal of the potentiating effect of choline is shown from an experiment on a single end-plate. In the upper part, a long choline pulse is applied during a series of brief *Ach* potentials. Before prostigmine there is an increase, after prostigmine a depression of the *Ach* potentials. In the lower part of the figure the effects are shown by imposing a short choline pulse during a steady *Ach* potential.

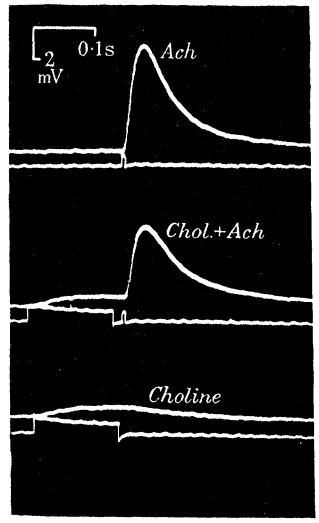

FIGURE 10. Inhibition of *Ach* potentials by choline, in prostigmine-treated muscle. A short *Ach* pulse preceded by a long choline pulse. Monitor calibration ('2 mV' scale): $1{\cdot}7 \times 10^{-8}$ A.

Thus, in the prostigmine-treated muscle choline has a 'curarizing' effect, like C_{10}. The potency and time course of this action are summarized in table 1, and compared with the action of *d*-tubocurarine (Castillo & Katz 1957*a*). Attention may be directed to two points: the inhibitory potency of the choline pulses is, roughly, one-twentieth of that of *d*-tubocurarine. The time course of decay, however, is relatively fast; if we compare it in each case with the decay of the *Ach* potential (elicited from the corresponding twin pipettes), we find that the half-decay of curarine inhibition is 35 times longer than that of the *Ach* potential, while for choline the factor is only about two.

As with C_{10}, a simple inhibitory effect was found in an experiment in which choline interacted with succinylcholine, even though no prostigmine was present.

The three actions, (i) weak depolarization, (ii) potentiation of *Ach* in normal Ringer solution, (iii) inhibition of *Ach* in the prostigmine-treated muscle, can be explained on our initial hypothesis as arising from a competitive interference with both receptors and esterase. The interpretation is very similar to that offered for

the triple effect of decamethonium. The difference between the two drugs is only quantitative, and concerns the potency and time course of their effects. With C_{10} there was evidence of an initial inhibitory action even in normal Ringer solution; with choline, we may suppose that the inhibitory action is normally concealed by

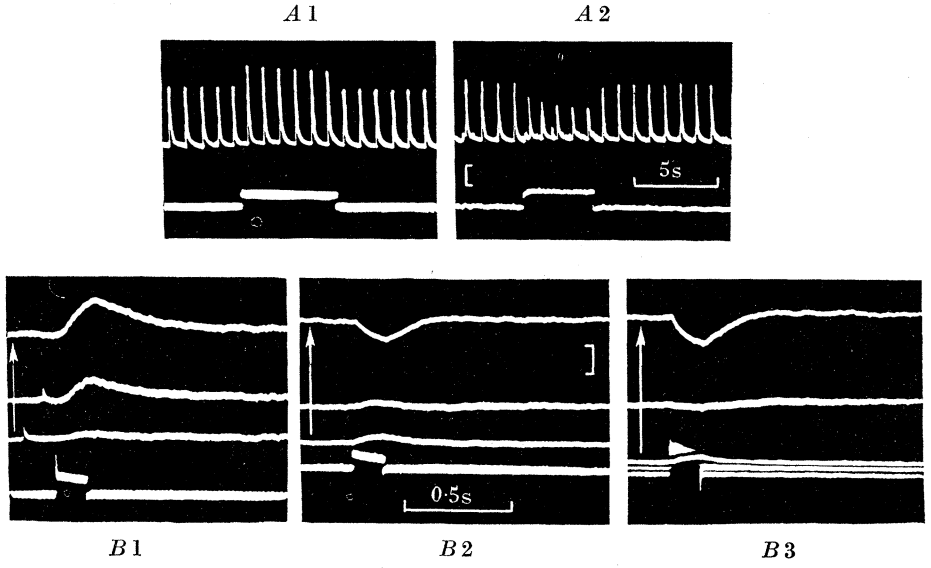

FIGURE 11. Choline/*Ach* interaction, before and after prostigmine treatment. All records were obtained from one end-plate. *A*, effect of choline on brief *Ach* potentials. Long choline pulse, shown in monitor trace. *A* 1 before, *A* 2 after prostigmine. Vertical scale: 2 mV, $1{\cdot}1 \times 10^{-8}$ A. *B*, effect of choline-pulses on steady *Ach* potentials. *B* 1 before, *B* 2 and *B* 3 after prostigmine. Steady depolarization (in the direction of the arrows) was produced by lowering 'braking current' in *Ach* barrel. Vertical scale: 2 mV (for *B* 2 and *B* 3), 1 mV (for *B* 1), $1{\cdot}7 \times 10^{-8}$ A (monitor).

TABLE 1. INHIBITORY ACTION OF CHOLINE, COMPARED WITH *DTC*

(Note that choline pulses were used to inhibit steady *Ach* potentials.)

	depolarization				inhibition			
agent	approx. pulse strength (C)	pulse depolarization (mV)	half-decay time (ms)	steady depolarization (mV)	agent	approx. pulse strength (C)	% inhibition	half-decay time (ms)
Ach or *Carb*	6×10^{-11}	5	43	—	*DTC*	4×10^{-10}	50	1400
Ach (+prostigmine)	4×10^{-11}	5	60	9	choline (+prostigmine)	4×10^{-9}	28	115

relatively fast and powerful depression of *Ach* hydrolysis. One may wonder whether the interaction here described can play a part in normal impulse transmission: this seems, however, very unlikely for the dose needed in these experiments exceeded that of *Ach* more than tenfold (see also Hutter 1952).

Discussion

The two main results described in this paper are (i) the potentiation of *Ach* by stable choline derivatives and (ii) the fact that a substance may, at the same time, exhibit depolarizing and curarizing properties. The last observation suggests the competitive formation of an inert drug-receptor compound, as an intermediate step in the depolarizing reaction.

The potentiation has been tentatively ascribed to a competitive combination between drug and *Ach*-esterase. The evidence for this view is based on the observations that potentiation diminishes or disappears (*a*) when the experiment is performed in the presence of a strong enzyme inhibitor, (*b*) when acetylcholine is replaced by a stable ester like carbachol or succinylcholine. This evidence is suggestive but indirect, and it would be desirable to correlate it with direct determinations of the anti-esterase potency of the different drugs, in similar short-term applications. Unfortunately, the available methods for measuring cholinesterase activity cannot easily be adapted to these conditions. Conversely, the present method of ionophoretic pulses can give results which do not correspond closely with those obtained by steady drug application. Consider, for example, a substance which has a high affinity to the enzyme and acts as a very potent inhibitor under equilibrium conditions, but whose reaction is very slow. In a short-term experiment of the present kind, such a drug may be less effective than a substance of lower final 'affinity', but higher rate constants. To illustrate this point, prostigmine and eserine which are very potent anti-esterases both act and dissociate slowly (Easson & Stedman 1936; Eccles, Katz & Kuffler 1942, figure 10; Augustinsson & Nachmansohn 1949; Goldstein 1951). We have tried to potentiate *Ach* potentials by ionophoresis from prostigmine-filled pipettes, but failed to do so and with strong pulses obtained an inhibitory action. The inhibitory effect as such was not surprising, for many anti-esterases are known to block *Ach* receptors in sufficient concentration (Fatt 1950; Fatt & Katz 1952; Paton & Perry 1953). But with the usual prolonged application, the inhibitory action of prostigmine on the end-plate response requires a larger dose than its potentiating effect (Eccles & McFarlane 1949), while it appears that with our pulse technique the relative potencies are reversed. The relative inefficacy of C_{10} as a depolarizing agent, which has been described in the previous paper (Castillo & Katz 1957*b*), may have a similar explanation.

To summarize, the interpretation of the potentiating effect as arising from inhibition of *Ach* hydrolysis cannot be regarded as definite and requires verification by a more direct, but similarly rapid method.

On the other hand, the inhibitory effect of weak or slow depolarizers seems to be satisfactorily explained by the hypothesis of an intermediate receptor compound (cf. Ariëns 1954; Ariëns & de Groot 1954; Ariëns *et al.* 1955). The next step in this reaction is still entirely speculative. In equation (2) it has been described as a reversible change of the kind $SR \rightleftharpoons SR'$, though various other arbitrary forms could have been chosen. It is clear that the two steps of equation (2) do not give an adequate description of all the changes which occur during an intense and pro-

longed *Ach* application. Recent studies by Thesleff (1955) and others have shown that the depolarization gradually disappears and the receptor mechanism becomes 'refractory' (see Castillo & Katz 1956, p. 165) if a high concentration of *Ach* in maintained for several minutes. To take this process into account, a third step might be added to equation (2), e.g.

$$Ach + R \overset{\text{(i)}}{\rightleftharpoons} AchR \overset{\text{(ii)}}{\rightleftharpoons} AchR' \overset{\text{(iii)}}{\longrightarrow} Ach + R'. \qquad (3)$$

The reversible reactions (i) and (ii) proceed at high rate, with initial formation of an inert (*AchR*) and subsequently of a depolarizing receptor compound (*AchR'*); reaction (iii), however, is a slow process by which the receptor is degraded into an inactive, and non-reactive, form (*R'*). This is not completely irreversible, but spontaneous recovery from *R'* to the initial reactive form (*R*) is very slow and has been omitted from the scheme. It would follow that with a maintained high concentration of *Ach*, the reactive groups become gradually exhausted and transformed from $R \rightarrow R'$, a process which is slowly reversed when *Ach* is removed from the bath.

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