Brit. J. Pharmacol. (1964), **23**, 575–591.

THE RELATIONSHIP BETWEEN ANTICURARE ACTIVITY AND TIME COURSE OF THE ENDPLATE POTENTIAL; A STRUCTURE-ACTIVITY APPROACH

BY

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(*Received March* 26, 1964)

A comparison of the effects of several quaternary ammonium ions has been made on the intracellularly recorded endplate potential of curarized frog muscle. The hydroxyanilinium ions usually caused an increase in endplate potential amplitude, a slowing of rate of rise and marked prolongation, but rarely caused spike generation. Contrariwise, tetraethylammonium and triethylphenylammonium consistently caused an increase in both amplitude and rate of rise of the endplate potential but no prolongation; these endplate potentials generated muscle spikes. The results suggest a relationship between rate of rise of the endplate potential and the probability of spike generation. Using the neurally evoked contractile response as a signal of transmitter action, the hydroxyanilinium ions were found to be relatively weak antagonists of tubocurarine at deep levels of curarization, indicating a ceiling effect. There was also a ceiling to the effect of hydroxyaniliniums on endplate potential amplitude. Thus, in the presence of high concentrations of tubocurarine, tetraethylammonium is a more potent anticurare agent than is triethyl(3-hydroxyphenyl) ammonium.

The endplate potential of curarized skeletal muscle is a sign of transmitter action at the vertebrate neuromuscular junction. Accordingly, modification of various parameters of this potential by chemical agents indicates changes either in the amount of transmitter available for interaction with the endplate membrane or in the nature of the interaction itself. Interest has centred mainly on two types of pharmacologic modifications of the curarized endplate potential: (1) increase in amplitude, and (2) decrease in rate of decay. The first effect is produced, for example, by tetraethylammonium ion (TEA) (Stovner, 1958; Koketsu, 1958) and guanidine (Otsuka & Endo, 1960) and has been attributed to increased transmitter release. The second effect is typically produced by anticholinesterase agents and has been classically associated with decreased rate of transmitter destruction and, hence, prolonged transmitter action (Fatt, 1959). With both types of endplate potential change, the important event for anticurare action is the achievement of an amplitude great enough to generate a conducted potential.

In addition to their effect on rate of decay, anticholinesterases increase endplate potential amplitude. If these compounds do not affect rate of rise, then amplitude increases to a higher but *later* peak. This is exactly what has been observed with

physostigmine (Eccles, Katz & Kuffler, 1942), neostigmine (Fatt & Katz, 1951) and edrophonium (Nastuk & Alexander, 1954). The problem raised by the present experiments is whether this type of endplate potential is invariably associated with a conducted response, even if the normal threshold firing level is reached. On purely physiologic grounds, rate of rise of a generator potential is at least as important a determinant of the conducted response as is amplitude.

These experiments were undertaken in order to clarify the relationship between various parameters of endplate potential and anticurare action. For this purpose, a structure-activity approach was adopted. With one exception, the compounds used in these experiments were ethonium ions, and these were selected for the following reasons: (1) ethonium ions lack direct depolarizing actions on excitable membranes, thus simplifying the interpretations of drug-induced changes in endplate potential; (2) these ions were previously shown to potentiate the neurally-evoked contractile response of amphibian skeletal muscle (Nastuk & Alving, 1959); and (3) these ions vary, according to structure of the molecule, from very weak to relatively potent anticholinesterase activity (Kuperman, Gill & Riker, 1961), thus permitting evaluation of the influence of this parameter on the endplate potential.

METHODS

All experiments were performed on the isolated sciatic nerve-sartorius muscle preparation of the frog (*Rana pipiens*) at room temperature (20 to 22° C). The muscle was initially bathed in Ringer solution containing (mM): 110.88 NaCl, 2.0 KCl, 1.8 $CaCl_2$, 0.1 NaH_2PO_4, and 2.02 $NaHCO_3$, and buffered to *p*H 7.2. The main portion of the nerve was mounted on bipolar platinum electrodes in a separate compartment containing light mineral oil. All compounds were dissolved in Ringer solution.

Electrical recordings. Transmembrane potentials were recorded with intracellular glass capillary microelectrodes filled with 3M-potassium chloride solution (Nastuk & Hodgkin, 1950). These electrodes had tip diameters of less than 0.5 μ and resistances of 10 to 15 M Ω. They were flexibly mounted at the end of a 0.25 mm chlorided silver wire and were connected to the input of a neutralized-input electrometer amplifier. Potentials were displayed on an oscilloscope and photographed with a Polaroid camera.

With the aid of a binocular microscope, superficial neuromuscular junctions were located in the pelvic zone of the muscle. Intramuscular branches innervating endplates in other regions of the muscle were cut.

Endplate potentials were developed in response to supramaximal rectangular-wave shocks of 0.01 msec duration delivered once every 30 sec to the nerve. Neuromuscular transmission was blocked after 30 min of treatment with D-tubocurarine chloride (3×10^{-6} g/ml.). Only those endplate potentials with amplitudes between 3 and 10 mV (average, 5 mV) were selected for studying anticurare activity. All drug solutions were introduced into the muscle bath through a 27 gauge needle, and tubocurarine was added to each solution to maintain a constant concentration in the muscle bath. Rapid distribution of added solutions was achieved by continuous and vigorous bubbling of air through the muscle chamber. Despite this mechanical agitation, microelectrodes were not displaced and the resting membrane potential maintained constant value for at least 20 min. Unless otherwise stated, each nerve-muscle preparation was discarded after determining the action of one concentration of a particular anticurare agent on a single curarized endplate potential.

Recording of contractile response. The muscle was mounted horizontally with the pelvic end tightly fixed to the floor of a paraffin chamber. The tendon at the femoral end was connected by silk thread to a strain gauge, the output of which was coupled to an ink-writing

oscillograph. Tension on the muscle was initially adjusted to 0.5 g, causing stretching to 130% of resting length. Supramaximal rectangular-wave shocks of 0.01 msec duration were applied to the nerve once every minute.

A 20 ml. syringe was connected by polyethylene tubing to the muscle chamber, and this chamber could be completely emptied of fluid and refilled within several seconds. Before beginning an experiment, the freshly dissected nerve-muscle preparation was mounted and allowed to equilibrate to tension adjustments and solution changes for a period of 30 min. After another 10 min period during which contractile responses of constant amplitude were recorded, the Ringer solution in the muscle chamber was replaced by a Ringer solution containing tubocurarine. After 30 min, this solution was replaced by one containing a known concentration of anticurare agent plus the original concentration of tubocurarine. The effect of this solution was then studied for another 30 min, after which the entire preparation was discarded.

Materials. The following quaternary ammonium ions were synthesized as iodides either in this laboratory or by Eastman Kodak: triethyl(3-hydroxyphenyl)ammonium (3-OH-TEPA), diethyl(3-hydroxyphenyl)methylammonium (3-OH-DEMPA), triethyl(4-hydroxyphenyl) ammonium (4-OH-TEPA), triethylphenylammonium (TEPA) and tetraethylammonium (TEA). Crystalline D-tubocurarine chloride (curare) was obtained from Mann Research Laboratories (N.Y.).

RESULTS

The major differences between the effects of TEA and 3-OH-TEPA on curarized endplate potentials are illustrated in Figs. 1, 2 and 3. The two major points of contrast relate to rate of rise and rate of decay.

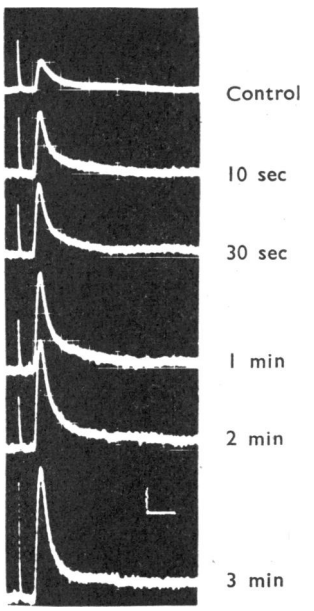

Fig. 1. Effect of TEA on curarized end-plate potential. From top to bottom: control, then 10 sec, 30 sec, 1 min, 2 min and 3 min after 10^{-4} M-TEA. Calibration marks: 5 mV and 5 msec. Resting potential: 96 mV.

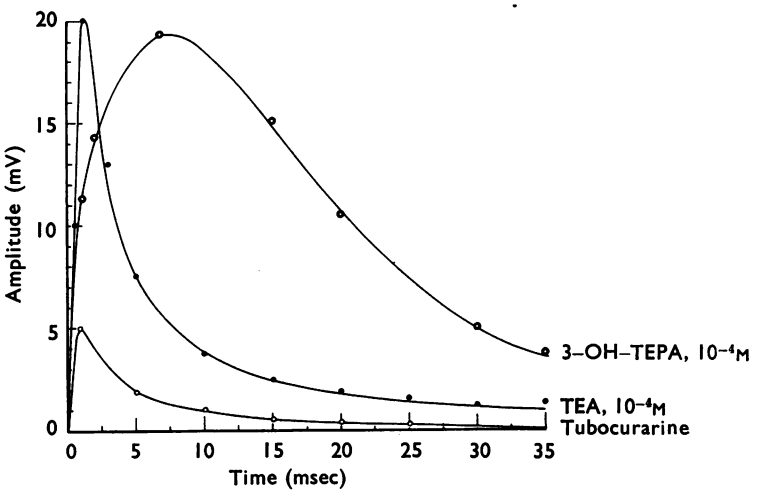

Fig. 2. Comparison of the effects of TEA and 3-OH-TEPA on the curarized end-plate potential. The graph for tubocurarine is the average of six end-plate potentials. Each of the other tracings is the average of three. The 3-OH-TEPA end-plate potentials used for averaging were maximal responses to the drug; those for TEA were recorded just before appearance of the action potential.

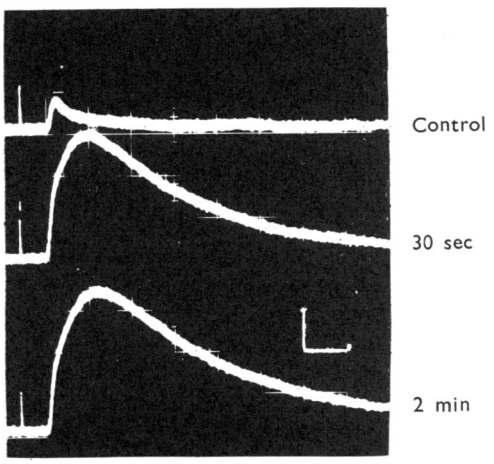

Fig. 3. Effect of 3-OH-TEPA on the curarized end-plate potential. From top to bottom: control, then 30 sec and 2 min after 10^{-4} M-3-OH-TEPA. Calibration marks: 5 mV and 5 msec. Resting potential: 89 mV.

The effect of TEA is characterized by an increase in amplitude and rate of rise. For example, in the experiment illustrated in Fig. 1, the rise time after 2 min of TEA action was only 1.3 msec, although the endplate potential amplitude at this time was about 4.5-times the control. The rise time of the control curarized endplate potential in this particular example was 1 msec. Thus, the rate of rise had increased markedly under the influence of TEA. In the endplates of ten

different curarized nerve-muscle preparations treated with 10^{-4} to 10^{-5} M-TEA, there were comparable increases in rate of endplate potential rise. This effect is also clearly shown in Fig. 2 in which three TEA endplate potentials are averaged and graphically presented.

Even with a flexibly mounted microelectrode, it was impossible to record consistently the muscle spike and endplate potential simultaneously. However, fibres in the pelvic region of the muscle were observed always to contract in response to nerve stimulation within 2 to 4 min after application of 10^{-4} to 10^{-5} M-TEA. In those preparations where the electrode was not displaced from the muscle fibre during contraction, the endplate potential amplitude was 19 to 24 mV at the onset of muscle spiking; this threshold firing level was achieved within 1 to 1.7 msec. The rate of repolarization was not slowed by TEA even at these spike generating amplitudes. In fact, a slight increase in repolarization rate usually occurred, probably due to a decrease in membrane time constant associated with intense depolarization.

The typical action of 3-OH-TEPA on the curarized endplate potential consists of an increase in amplitude, a marked slowing of rate of decay, and a slowing of rate of rise. This last action is particularly striking and stands in distinct contrast to the effect of TEA. For example, in the experiment of Fig. 3, peak endplate potential amplitude just 30 sec after application of 3-OH-TEPA was not reached until 6.5 msec after the endplate potential origin, a rise time almost six-times longer. However, it should be clear from Fig. 3 and especially from Fig. 2 that the action of 3-OH-TEPA on endplate depolarization is more complex than appears from measurement of rise time or rate of rise. During the first msec of transmitter action in the presence of this ethonium ion, there usually occurs an *increase* in rate of rise, just as in the presence of TEA. Subsequently, there is a slowing of the rate of rise so that the endplate potential reaches maximum amplitude in the form of a hump rather than a sharp peak. This hump is also caused by the pronounced slowing of rate of decay.

The type of response to 3-OH-TEPA described above was observed in the endplates of thirteen preparations treated with 10^{-4} to 5×10^{-5} M of the drug. Peak endplate potential amplitudes at time of maximal drug effect varied from 18 to 23 mV. It is noteworthy that muscle spikes were not generated by these enormously prolonged and slow rising endplate potentials although their amplitudes were within the range required for spike generation in the presence of TEA. Even the addition of more 3-OH-TEPA to the muscle bath did not cause these endplate potentials to trigger action potentials, but rather resulted in further slowing of rate of rise and depression of endplate potential amplitude. However, after application of 10^{-4} to 10^{-5} M of the drug, nerve stimulation produced contractions of muscle fibres in the pelvic region; this indicates that a certain population of endplates were developing potentials of a time course and/or amplitude different from the endplate potentials actually recorded.

No alteration of transmembrane resting potential was produced by 10^{-4} M of either TEA or 3-OH-TEPA after 15 min of treatment. In this respect, both drugs

are typical of ethonium ions, lacking significant depolarizing action on excitable membranes.

The effect of gradually increasing concentrations of 3-OH-TEPA on the curarized endplate potential. This was determined with the initial concentration of ethonium ion in the muscle bath 10^{-5} M, which was increased a hundredfold over a period of 15 min. Accordingly, the endplate responses measured during this time were both concentration- and time-dependent. The object of this method of drug application was to avoid any possible depressant action of the compound, thus permitting curarized endplate potentials to achieve threshold firing level and generate propagated action potentials. As can be seen in the tracings from a typical experiment (Fig. 4), this objective was not fulfilled . Even after total concentration as high

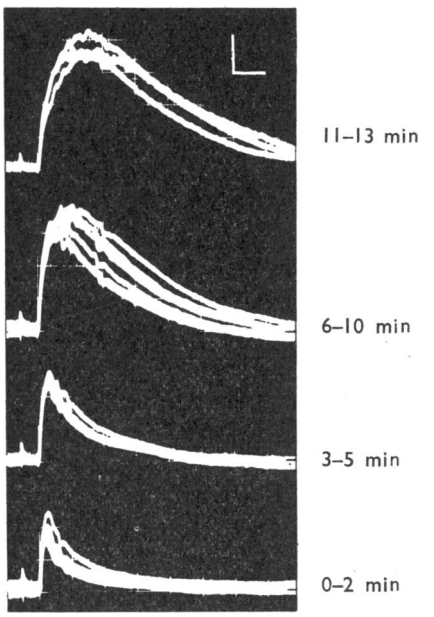

Fig. 4. Effect of gradually increasing concentrations of 3-OH-TEPA on the curarized end-plate potential. From bottom to top: control, then beginning of 3-OH-TEPA infusion with 10^{-5} M, next tracing recorded 30 sec later, then 1.5 min later and at 1 min intervals thereafter. The concentration at the end of 2 min was 5×10^{-5} M, at 5 min 10^{-4} M, at 10 min 5×10^{-4} M, and at 13 min 10^{-3} M. Calibration marks: 5 mV and 5 msec. Resting potential: 98 mV.

as 10^{-3} M had been reached, muscle spikes were not generated by prolonged endplate potentials of at least 20 mV amplitude. Again, it is important to note that the peak values of these endplate potentials at the times of maximal drug effect were not attained until 6 msec or more after onset.

Certain additional features of these results are of interest and are illustrated in the tracings of Fig. 4: (1) in the presence of 10^{-5} M-3-OH-TEPA, the rate of endplate potential rise remains unaltered although amplitude increases; (2) after a concentration of about 5×10^{-4} M has been reached, the rate of rise slows

significantly; (3) the rate of decay begins to slow noticeably at the same time as the rate of rise slows, although the endplate potential amplitude is approximately doubled before this time.

The effects of closely spaced nerve stimuli. These were studied on curarized preparations in the presence of 10^{-4} M-3-OH-TEPA. The purpose of these experiments was to determine whether a second endplate potential, produced within a few msec after the first, would generate a conducted potential even if the first one did not. The possibility was considered that 3-OH-TEPA might exert a stabilizing action on the curarized endplate potential which causes a decreased rate of endplate potential rise and that this action, in turn, might be associated with the absence of spike generation. A depressant effect on the electrogenic membrane surrounding the endplate region was also considered. The results of the double stimuli experiments fail to support these possibilities. For example, note in Fig. 5 that a second

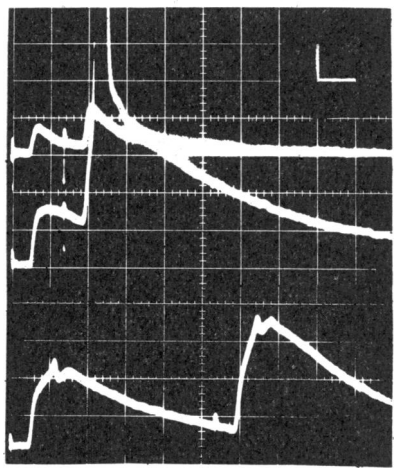

Fig. 5. Effect of 3-OH-TEPA in conjunction with double stimuli. Top: control, 7 msec interval between stimuli; middle: 30 sec after 10^{-4} M-3-OH-TEPA, 7 msec interval; bottom: 1 min later, 27 msec interval. Although not shown, double stimuli at a 7 msec interval initiated muscle spikes as in middle tracings. Calibration marks: 10 mV and 5 msec. Resting potential: 85 mV.

endplate potential produced 7 msec after the first is facilitated and generates a propagated response. In other experiments this same type of response to the second of two closely spaced nerve stimuli was observed, and in every case the first endplate potential reached a 20 mV peak, was greatly prolonged and had the long rise time of 6 to 8 msec.

Generation of muscle action potentials by curarized endplate potentials. This effect after application of 10^{-4} M-3-OH-TEPA is illustrated in Fig. 6. At 2 min after drug application, the endplate potential amplitude is more than twice that of the control but there is no slowing of the rates of rise and decay. In the 3 min tracing, an action potential is initiated at 3 msec after the endplate potential origin.

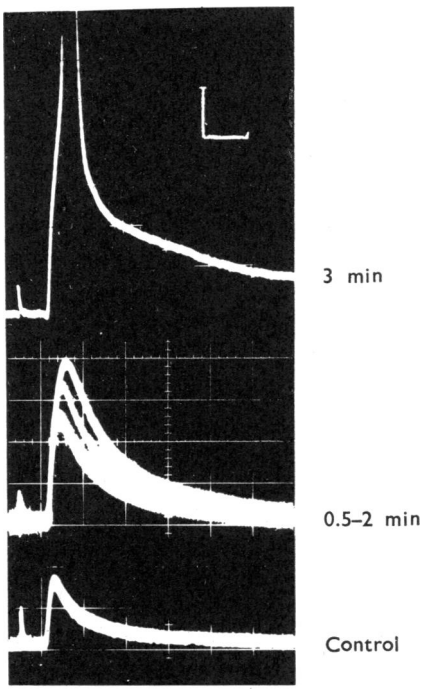

Fig. 6. Effect of 3-OH-TEPA on the curarized end-plate potential. From bottom to top: control, then 30 sec, 1 min, 2 min and 3 min after 10^{-4} M-3-OH-TEPA. Calibration marks: 5 mV and 5 msec. Resting potential: 90 mV.

The rate of rise of this spike-generating endplate potential is only slightly faster than that of control. The falling phase of the action potential appears prolonged, probably because the rate of endplate potential decay is slowed at this time ; but this slowing seems quantitatively trivial compared to that observed in previously described endplate potentials (for example, Fig. 3). The response to 3-OH-TEPA illustrated in Fig. 6 is similar to that produced in the endplates of five preparations. In each of these, the threshold firing level was 20 to 26 mV and was attained within 2 to 4.5 msec after the endplate potential origin ; there was a relatively insignificant slowing of rate of decay.

In Fig. 7 there is illustrated an atypical circumstance in which application of 3-OH-TEPA produces spike generation. This type of response is probably associated with an abnormally low resting transmembrane potential of the muscle fibre and, hence, a low threshold for spike generation. Nevertheless, there are certain interesting aspects to this response. Just 30 sec after application of 3-OH-TEPA, an action potential is initiated from a 15 mV endplate potential. The rate of rise at this time is unaltered but becomes faster in subsequent tracings. At 4 min, the rate of endplate potential rise is almost as fast as that of the action potential, so that the level of impulse initiation is difficult to determine. In the 30 sec and 1 min tracings, there is probably some prolongation of endplate potential as evidenced by the slow falling phase of the action potential. At 2 min a striking

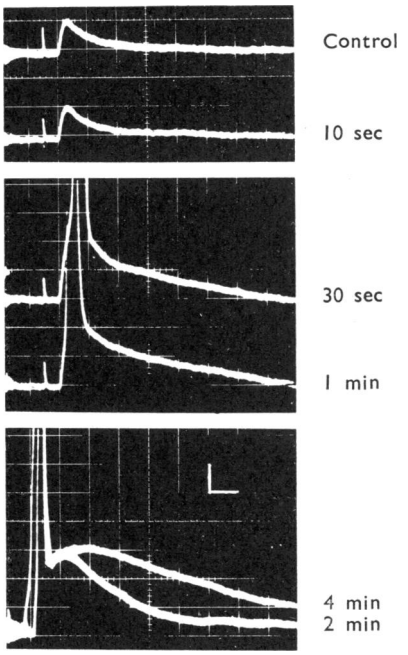

Fig. 7. Effect of 3-OH-TEPA on the curarized end-plate potential. From top to bottom: control, then 10 sec, 30 sec, 1 min, 2 min and 4 min after 10^{-4} M-3-OH-TEPA. Calibration marks: 5 mV and 5 msec. Resting potential: 75 mV.

phenomenon occurs; a distinct hump appears on the tail of the action potential. This "secondary" slow potential increases in amplitude and duration with the passage of time (4 min tracing), and is similar to the prolonged and slowly rising endplate potentials typically associated with the action of 3-OH-TEPA. In this particular experiment, however, the point of interest is that the slow potential appears much later than the muscle spike during the time/action curve of 3-OH-TEPA.

The effect of 10^{-4} M-TEPA on the curarized endplate potential. This was studied in six preparations. In each case the action was similar to that illustrated in Fig. 6, and muscle spiking was consistently produced from firing levels of 21 to 25 mV. Unlike its *meta*-hydroxy analogue, this ion does not produce endplate potential prolongation, and unlike TEA, it does not speed the rate of rise.

The effects of 4-OH-TEPA on the curarized endplate potential. These were similar to those described earlier for 3-OH-TEPA (Figs. 3 and 4). In two preparations, 10^{-3} to 10^{-4} M of the *para*-hydroxy analogue greatly slowed both the rate of rise and the rate of decay. Simultaneously, the endplate potential amplitude increased, but even at a concentration of 10^{-3} M the maximal amplitude achieved was 15 mV, far below the threshold firing level. There appeared to be a comparatively low ceiling to the action of 4-OH-TEPA on endplate potential amplitude, and this point requires further investigation.

The action of 3-OH-DEMPA on the curarized endplate potential. This was investigated in five preparations. This compound is of great interest in connection with the present experiments because it is a more potent cholinesterase inhibitor than its ethonium analogue (Smith, Cohen, Pelikan & Unna, 1952 ; Kuperman *et al.*, 1961). Like its ethonium analogue, this ion has no depolarizing action in concentrations up to 5×10^{-4} M. The typical endplate potential effects of 3-OH-DEMPA (Fig. 8) are similar to those already described for the ethonium analogue, namely a decrease in rate of rise and rate of decay. However, the effects of 3-OH-DEMPA

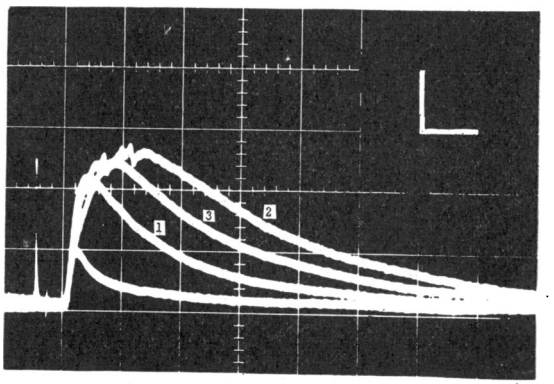

Fig. 8. Effect of 3-OH-DEMPA on the curarized end-plate potential. From bottom to top: control, then 1 min (1) and 2 min (2) after 10^{-4} M-3-OH-DEMPA; then the concentration was increased to 5×10^{-4} M and the response recorded 1 min later (3). Calibration marks: 6 mV and 5 msec. Resting potential: 90 mV.

on endplate potential amplitude is more similar to that of 4-OH-TEPA in that a low ceiling exists, and the effect is biphasic. Thus, in the presence of 10^{-4} M-3-OH-DEMPA, the peak endplate potential amplitude in four preparations did not increase above 15 mV and, accordingly, spike generation was not observed in these instances. The replacement of the 10^{-4} M solution by a more concentrated 3-OH-DEMPA solution, as in the experiment of Fig. 8, caused a greater decrease in rate of rise and lowering of endplate potential amplitude. With the passage of time, both of these effects become intensified.

In one preparation in which 10^{-4} M-3-OH-DEMPA caused spike generation, rise time to firing level was only 2 msec and endplate potential amplitude was 20 mV. A secondary slow potential, similar to that illustrated in Fig. 7, was also observed.

The effect of tubocurarine. This was studied on the intracellularly recorded endplate potential in order to confirm one component of the drug's action which has long been known (Eccles *et al.*, 1942), namely slowing of rate of endplate potential rise. The reduction of endplate potential amplitude by tubocurarine has always been viewed as a primary effect; but proper interpretation of the effects of anticurare agents requires the recognition of both components of tubocurarine action. In each of these experiments the response of the endplate membrane to a nerve stimulus was first recorded in normal Ringer solution. Then a small volume of concentrated

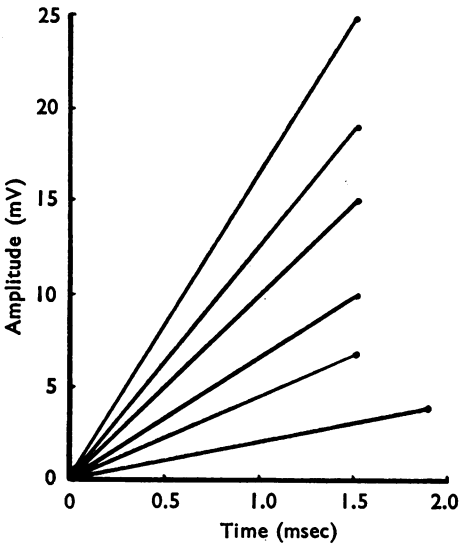

Fig. 9. Effect of tubocurarine on the end-plate potential. Tracings obtained from a typical experiment are plotted in order to show changes in amplitude and rise time with increasing concentrations of tubocurarine. Only the rising phase of each end-plate potential is represented. The maximum point on each line is the peak end-plate potential amplitude. The topmost line represents the rising phase before tubocurarine. Then 1.5×10^{-6} g/ml. was added to bath. The next line represents the 2 min effect. The tubocurarine concentration then gradually increased over a period of 8 min; each succeeding line represents the effect at 2 min intervals. The final concentration, the effect of which is represented by the bottom line, was 3×10^{-6} g/ml. The resting potential remained constant throughout the experiment at 95 mV.

tubocurarine solution was infused slowly into the muscle bath over a period of 6 to 10 min until the tubocurarine concentration in the bath reached 3×10^{-6} g/ml. Endplate potential recordings were taken periodically throughout this procedure. In Fig. 9 the rising phases of normal and curarized endplate potentials recorded during a typical experiment are shown graphically. With increasing depth of curarization, the peak endplate potential amplitude becomes smaller and the rate of rise becomes slower; at maximal depth of curarization shown in Fig. 9, the rise time also becomes longer.

Effects of ethonium ions and 3-OH-DEMPA. These were measured on the neurally evoked contractile response of curarized preparations. This seems a useful approach to the proper evaluation of drug effects on curarized endplate potentials because the total number of endplate potentials sampled in this study with any one compound constituted a trivial fraction of the total population available, and the responses of curarized endplate potentials to drugs were highly variable, particularly for 3-OH-TEPA.

The concentration of tubocurarine used for the endplate studies (4.29×10^{-6}M or 3×10^{-6} g/ml.) was selected because it produced a complete paralysis of neuromuscular transmission, thus facilitating the impalement of muscle fibres by micro-

electrodes. In order to study the anticurare actions of drugs, using contractile response rather than endplate potentials as a signal of transmitter action, certain preliminary experiments were necessary. First, the dose/response relationship for tubocurarine was determined with concentrations ranging from 2×10^{-7} to 2×10^{-6} g/ml. The calculated regression line expressed a linear relationship between log concentration and percentage depression of contraction ($P<0.05$). The contractile responses were measured before and after a 30 min period of treatment with tubocurarine, the same duration of treatment used in the endplates studies. From the calculated regression line, it was determined that 1×10^{-6} g/ml. of tubocurarine produces a 66% depression of the contractile response. In the presence of this

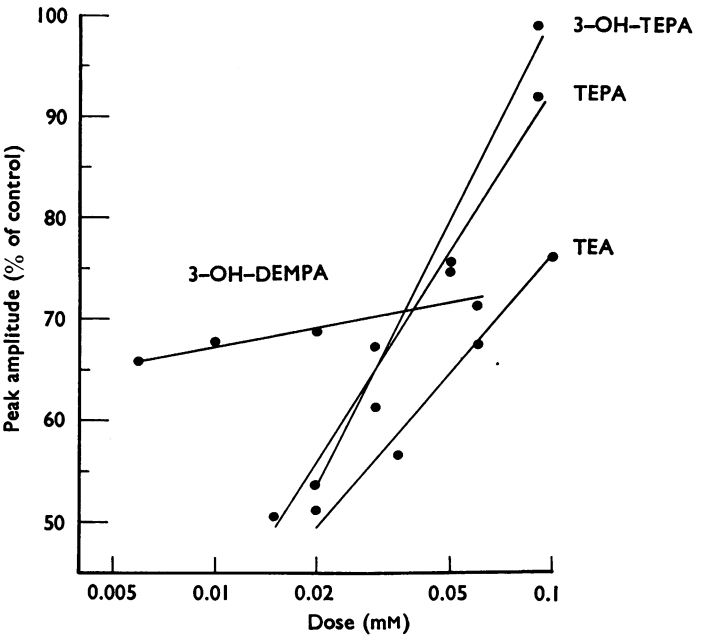

Fig. 10. The relationship between the dose of anticurare agent and the increase in contractile response. Only points below pretubocurarine level of contraction amplitude are shown. Each point is mean of nine observations.

concentration of tubocurarine, the relationship between dose and percentage increase in contractile response was determined for several anticurare drugs (Fig. 10). In the appropriate concentration, each drug previously discussed with regard to effects on curarized endplate potentials increased the contractile response to control (pretubocurarine) level. The calculated regression lines are all significantly linear ($P<0.05$), and the slopes of the calculated regression lines for 3-OH-TEPA, TEPA and TEA are not significantly different from each other ($P>0.1$). Amongst these three ions, the order of potency to increase contractile response to pretubocurarine level is 3-OH-TEPA>TEPA>TEA. The slope of the dose/response regression line for 3-OH-DEMPA is significantly less than the slopes of the other lines ($P<0.05$).

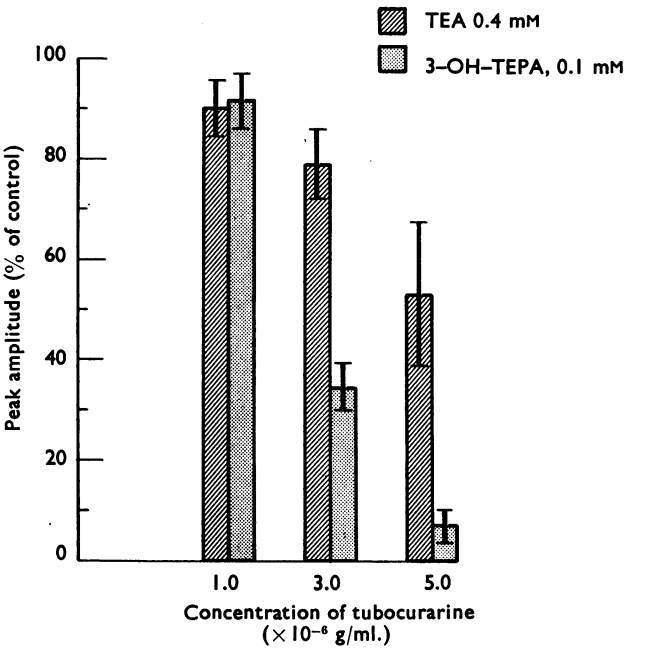

Fig. 11. Comparison between the effects of TEA and of 3-OH-TEPA on the contractile response in the presence of different concentrations of tubocurarine. The level of contractile response shown by each bar is mean of nine observations. The standard error is indicated by the line drawn through each bar. Respcnses to ethonium ions at 1×10^{-6} g/ml. of tubocurarine are not significantly different ($P > 0.7$). At 3 and 5×10^{-6} g/ml., the ethonium ion responses are significantly different ($P < 0.001$).

According to the calculated regression lines, equivalent degrees of antagonism of tubocurarine are produced by 0.4 mM-TEA and 0.1 mM-3-OH-TEPA (Fig. 11). Not only are these doses equipotent, but they also cause the depressed contractile response to increase to within 10% of pretubocurarine amplitude. Accordingly, these concentrations were selected for further study in the presence of higher concentrations of tubocurarine, that is the concentration used in the endplate experiments (3×10^{-6} g/ml.) and also 5×10^{-6} g/ml. The results of these experiments are graphically expressed in Fig. 11. It is apparent that with increasing concentrations of tubocurarine the action of 3-OH-TEPA on the contractile response is depressed to a far greater extent than is that of TEA. It is especially significant that, in the presence of 5×10^{-6} g/ml. of tubocurarine, TEA (0.4 mM) was an effective anticurare agent whereas the average response to 0.1 mM-3-OH-TEPA was minimal; in fact, there was no response to this concentration of 3-OH-TEPA at all in over one-half of the preparations tested.

In order to establish whether a true ceiling to the anticurare action of 3-OH-TEPA exists, its concentration was increased five-fold. The effect on contractile response of 0.5 mM of this ion in the presence of 5×10^{-6} g/ml. of tubocurarine was not

significantly different from that of 0.1 mM-3-OH-TEPA ($P>0.1$). Thus, at relatively deep levels of curarization, TEA is a more potent anticurare agent than is 3-OH-TEPA.

DISCUSSION

Perhaps the most obvious result of this investigation is that equimolar concentrations of structurally related quaternary ammonium ions have dissimilar effects on the curarized endplate potential. This result was unexpected because a careful survey of the literature pertaining to this field (see Werner & Kuperman, 1963) fosters the impression that the action of hydroxyanilinium ions and other cholinesterase inhibitors is characterized only by endplate potential prolongation, and that compounds like TEA increase endplate potential amplitude without causing prolongation. On the basis of the present experiments, it is difficult to characterize the effect of ethonium ions on neuromuscular transmission let alone the effect of the heterogeneous group of molecular structures which comprise the anticholinesterases. Even one compound, for example 3-OH-TEPA, produces more than one type of change in the curarized endplate potential. In fact, it seems clear that the number of pharmacologically induced endplate potential alterations observed depends to a great extent on the number of muscle fibres sampled and the number of compounds tested.

Of the five compounds used in these experiments, only TEA and TEPA consistently caused spike generation by curarized endplate potentials, and the hydroxyanilinium ions occasionally produced this effect. In each of these instances, endplate potential amplitude reached firing level within 2 to 4.5 msec; there was no slowing of the rate of endplate potential rise and no prolongation of endplate potential time course. In most experiments the hydroxyanilium ions did not produce spike generation even although endplate potential amplitudes were achieved which, in normal or TEA-treated muscles, were threshold levels. In those endplate potentials which did not trigger muscle spikes, peak amplitude was attained within 5 to 8.5 msec, and the rate of decay was greatly slowed. Thus, in addition to amplitude, the rate of endplate potential rise appears to be an important determinant of spike generation. Prolongation by itself seems to be of little consequence in this regard. Indeed, marked slowing of the rate of endplate potential decay is rarely associated with the appearance of conducted potentials. Perhaps the failure of the slowly rising endplate potential to trigger conducted potentials is caused by an accommodation of the electrogenic membrane to a slow outwardly directed current from the endplate sink; consequently, this membrane does not become depolarized to a threshold level.

It is well established that the current strength required for impulse conduction in axons and muscle fibres increases with decreasing rates of current rise (see Katz, 1939). However, there have been no experiments on the relationship between rate of rise of a synaptic potential like the endplate potential and the probability of impulse initiation. Hence, the present conclusion concerning the failure of slowly rising endplate potentials to generate spikes can only be tentative. In this connection, it is significant that ionophoretic application of acetylcholine to the endplate region evokes endplate potentials of 40 to 50 mV amplitude and very slow rates of

rise and decay; these giant endplate potentials rarely generate muscle action potentials (Castillo & Katz, 1955). The high threshold for impulse generation by these acetylcholine potentials could also be explained by accommodation of the electrogenic membrane to slowly rising endplate current.

Using the contractile response as a sign of transmitter action, other investigators have established a positive correlation between anticurare potency and anticholinesterase activity of many different compounds, including hydroxyanilinium ions (Blaschko, Bülbring & Chou, 1949; Smith *et al.*, 1952; Nastuk & Alving, 1959). On this basis, the present results seem paradoxical, for the most potent cholinesterase inhibitors are observed to be the most inefficient anticurare agents. However, these experiments also suggest a reason for the apparent discordance, namely that a lower concentration of tubocurarine is used for measurement of the contractile response than for measurement of intracellularly recorded endplate potentials. Because of the steepness of the tubocurarine dose/response regression line, even relatively small concentration differences are significant, and in this case the difference is about two- or three-fold. After enough tubocurarine has been applied to block contraction of every fibre in the muscle, endplate potential amplitude can be further decreased by higher concentrations of tubocurarine and, more importantly, the rate of rise also decreases. Significantly, most of the endplate potentials selected for study in these experiments were of low amplitude (3 to 7 mV) and slow rate of rise. The hydroxyanilinium ions cause an increase in endplate potential amplitude but not in rate of rise, at least not beyond the first msec of transmitter action. These ions usually cause an even further decrease in rate of rise so that the peak amplitude is attained after an excessive period of time. Contrariwise, TEA invariably produces an increase in rate of rise so that the peak endplate potential amplitude is reached within 2 msec. These effects on endplate potential time course and amplitude can be used to account for the differences between the anticurare actions of ethonium ions in the contraction experiments: the fact that at low concentrations of tubocurarine 3-OH-TEPA is the more potent anticurare agent and that, at higher concentrations of tubocurarine, TEA becomes more effective.

If we assume that 3-OH-TEPA and 3-OH-DEMPA possess only anticholinesterase actions at the neuromuscular junction, the present results suggest that such action is inefficient against relatively deep levels of curarization, regardless of dose. The more potent anticholinesterases, physostigmine and neostigmine, are no more efficient than the hydroxyanilinium ions in this respect (Kuperman & Okamoto, unpublished). With contraction as the criterion of response, the ceiling to the anticurare action of hydroxyanilinium ions has been explained on the basis of accommodation to slowly rising endplate current in a large population of muscle fibres. On the basis of the presently accepted theory of neuromuscular transmission, the production of slow-rising and prolonged endplate potentials by anticholinesterase agents acting on a deeply curarized muscle is entirely expected. One should also expect these endplate potentials to trigger action potentials at some amplitude, albeit much greater than normally required. However, there is a ceiling to the effect of hydroxyanilinium ions on endplate potential amplitude and, in fact, biphasic effects have been observed. The cause of this ceiling effect on endplate potential

amplitude is not known but it is possible, especially with relatively high concentrations, that these compounds have various types of depressant action at the neuromuscular junction. It is pertinent to note, for example, that closely related structural analogues of hydroxyanilinium ions depress neuromuscular transmission in the cat with no sign of potentiation at any dose (Kuperman *et al.*, 1961). Experiments on directly stimulated frog muscle indicate that the hydroxyanilinium ions do not depress excitability properties of the muscle fibre (Okamoto, 1964).

Another explanation for the ceiling effect of hydroxyanilinium ions on endplate potential amplitude relates to the amount of transmitter available for release by a single nerve impulse. Assuming that the only action of tubocurarine is to prevent reaction of endplate receptors with acetylcholine, it is possible that the nerve terminals cannot release the relatively large amount of acetylcholine required to depolarize the deeply curarized endplate membrane to threshold level, regardless of the duration of transmitter action. In conjunction with close interval paired nerve stimulation, endplate potentials of threshold amplitude are consistently evoked after application of hydroxyanilinium ions; the second of each pair of endplate potentials is facilitated, indicating increased availability of transmitter (Hubbard & Schmidt, 1963).

Mammalian experiments have revealed a potent and direct action of tubocurarine on the motor nerve terminals (Riker, Werner, Roberts & Kuperman, 1959a; Standaert, 1964), and it is possible that tubocurarine also has a presynaptic action at the amphibian neuromuscular junction. Although the nature of this presynaptic effect is not yet known, it is probably associated with decreased spike amplitude in the nonmyelinated axon terminals and decreased transmitter release. Such an action must obviously be considered in accounting for the observed ceiling to the anticurare activity of hydroxyanilinium ions. This is especially so if the primary site of hydroxyanilinium ion action is the same presynaptic focus which is suppressed by tubocurarine (Riker *et al.*, 1959b; Werner, 1960; Werner & Kuperman, 1963).

The actions of TEA and TEPA on neuromuscular transmission stand in marked contrast to those of 3-OH-TEPA and other hydroxyanilinium ions. Even in the presence of a completely paralysing concentration of tubocurarine, there is no ceiling to the effect of TEA or TEPA on endplate potential amplitude. The mechanism of action of TEA on the curarized frog neuromuscular junction has been studied by Koketsu (1958). From recordings of electrical activity in the most distal accessible branches of the motor axon, Koketsu concluded that TEA increases amplitude and duration of the negative afterpotential in the axon terminals. Presumably, this causes an increase in the amount of transmitter released by each nerve impulse. Increased transmitter release by TEA can account for the capacity of this ion to speed the rate of rise of the curarized endplate potential.

On the basis of our experiments, we may suppose that the presynaptic focus of action of TEA, and perhaps also of TEPA, is relatively insensitive to tubocurarine. Quite oppositely, the effect of 3-OH-TEPA is severely limited by high concentrations of tubocurarine. On those few occasions when 3-OH-TEPA produced a fast rising and spike generating endplate potential, a TEA-like action on the presynaptic terminals may have occurred.

This investigation was carried out with the aid of a U.S. Public Health Service Grant (NB-3597). It formed part of the work submitted by M. Okamoto for the Ph.D. degree at Cornell University during the tenure of a Predoctoral Fellowship, Grant 2G-99, U.S. Public Health Service.

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