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Actions of bretylium tosylate at the neuromuscular junction

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Summary

1. The actions of bretylium tosylate on neuromuscular transmission in the rat phrenic nerve diaphragm preparation have been investigated by electrophysiological methods. Additional experiments have been made on the effect of the drug on the frog rectus preparation and on the acetylcholinesterase of erythrocytes.

2. After bretylium, there was a reduction in the amplitudes of miniature endplate potentials (mepps), endplate potentials (epps) and acetylcholine potentials recorded in the diaphragm, and also in the contractures of the rectus in response to acetylcholine (ACh) and to carbachol.

3. After bretylium, there was a prolongation of the time courses of epps and ACh potentials. Under certain circumstances there was enhancement of the amplitudes of epps and ACh potentials and of the contractile responses to ACh but not to carbachol.

4. Bretylium reduced the velocity of hydrolysis of ACh by erythrocyte ghosts. This inhibition was competitive and the Ki of bretylium was 0·053 mM.

5. Bretylium did not cause a reduction in the mean quantal content of the epp in junctions blocked with Mg^{++}.

6. It is concluded that bretylium exerts both facilitatory and inhibitory influences on neuromuscular transmission, which, exerted simultaneously, may give the false appearance that the drug has little action at this synapse.

Introduction

The mode of action of adrenergic neurone blocking agents at adrenergic synapses is unknown, perhaps because the application of the electrophysiological techniques used in the analysis of synaptic mechanisms is difficult at these synapses. It was considered that an electrophysiological analysis of the effect of bretylium at the neuromuscular junction might be relevant to the problem as it has been reported that this drug acts presynaptically to block transmission (Dixit, Gulati & Gokhale, 1961). A preliminary account of some of this work was given to the British Pharmacological Society in January 1967.

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Methods

The phrenic nerve diaphragm preparation was dissected from white rats weighing about 200 g and of either sex, which had been killed by a blow on the head with subsequent section of the spinal cord in the neck. Either the left or the right hemidiaphragm was used, being immersed in saline of the following composition (mM): NaCl, 137 ; KCl, 5 ; $CaCl_2$, 2 ; $MgCl_2$, 1 ; NaH_2PO_4, 1 ; $NaHCO_3$, 12 ; dextrose, 25, and gassed with 5% CO_2 in O_2. For electrical recording, the diaphragm was pinned over a Perspex dome in a 20 ml bath and saline was made to flow through the bath at approximately 1 ml/minute. Experiments were carried out with the saline at room temperature or maintained at 36° C.

Intracellular recordings were made with glass microelectrodes filled with 3 M KCl. Acetylcholine (ACh) or carbachol (Carb) was applied to endplates by electrophoretic ejection from single or double barrelled micropipettes filled with a 2 or 3 M solution of these substances. The ejecting pulse was provided by a circuit with an output series resistor of 100 MΩ and with provision for backing current (Curtis, 1964).

Extracellular records were made with insulated silver wire electrodes (0·127 mm diam., diamel coated ; Johnson, Matthey & Co. Ltd.). One electrode was positioned at the endplate region which was located in unblocked preparations as the point along the muscle fibre at which the spike potential was diphasic and had minimal latency, and in curarized preparations, where the endplate potential had maximal amplitude and rate of rise ; at this point a presynaptic spike was often also recorded. The phrenic nerve was stimulated through a pair of Ag electrodes mounted in a Perspex tube. The indifferent recording electrode was suitably positioned in the bath away from the diaphragm to minimize the stimulus artifact. The bandwidth of the recording system was 10 kHz–0·2 Hz (−3db).

Experiments were done on the frog rectus preparation using a technique based on that described by MacIntosh & Perry (1950). Acetylcholine or carbachol was kept in contact with the preparation for 2·5 min at 15 min intervals. The rectus was exposed to drugs 15 min before the agonist was added.

The drugs used were bretylium tosylate, a gift from Burroughs Wellcome & Co. Ltd. for which we are grateful, D-tubocurarine chloride, carbachol and acetylcholine chloride.

Results

Effect of bretylium on miniature endplate potentials

Miniature endplate potentials (mepps) were recorded using intracellular microelectrodes in eleven preparations at room temperature, that is at about 20° C. The amplitude of mepps was always reduced within 5 min after the addition of bretylium to the bath to give a concentration of 0·49 mM. In some cells, mepps were abolished, but in three cells the mepps although reduced in amplitude, were still measurable. In these experiments, one of which is shown in Fig. 1, there was no reduction of the membrane potential after bretylium, nor was there any change in the mean number of mepps recorded in each oscilloscope sweep lasting 2 s, which suggests that the frequency of spontaneous discharge of quanta was not altered. The reduction of the amplitude of mepps was reversed when the preparation was

washed. When a lower concentration of bretylium was used, the mepps were again reduced in amplitude but to a smaller extent (Figs. 1, 2). In some experiments mepps were recorded at a higher sweep speed ; after the addition of bretylium, they were reduced in amplitude and also had a longer time course. The time course of potentials is represented by the duration at half amplitude, that is the time between the rising and falling phases of the potential measured at half the peak amplitude. After bretylium, 0·12 mM (in Fig. 2), the duration at half amplitude of the mepps was increased to 1·49 of the control.

At 36° C bretylium in a concentration of 0·49 mM always reduced the amplitude of mepps to unrecordable levels.

Effect of bretylium on the endplate potential

Endplate potentials (epps) were recorded intracellularly from muscle cells after neuromuscular transmission had been blocked in several ways.

Bretylium block. Neuromuscular transmission was blocked at room temperature following the addition of bretylium in concentrations of 1–2 mM. In nine preparations blocked in this way, no mepps were recorded but epps were recorded when the

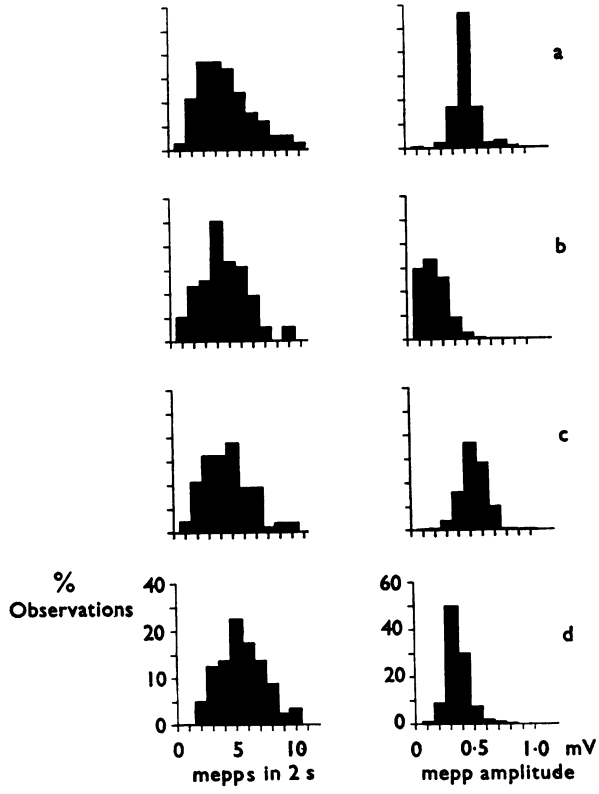

FIG. 1. Rat diaphragm, room temperature, intracellular recording from one cell with membrane potential 60 mV. Left: histograms showing the number of mepps recorded in each sweep of 2 s duration. Histograms compiled from 60–100 sweeps. Right: histograms showing the amplitudes of mepps. Each histogram compiled from 250 to 300 mepps. (a) Control conditions ; (b) recording begun 5 min after the addition of bretylium to give a concentration of 0·49 mM ; (c) recording begun 10 min after washing the preparation ; (d) recording begun 5 min after adding bretylium, 0·24 mM.

phrenic nerve was stimulated. The duration at half amplitude of epps in the bretylium blocked preparations was about three times that of epps recorded in curarized preparations and varied from 5·5 to 16 ms.

With repetitive stimulation at frequencies higher than 0·1 Hz, the epps in the bretylium blocked preparation decreased progressively in amplitude. With stimulation at 1 Hz the epp diminished to about half the control size, and at 10 Hz to about 0·28 of the control. Repetitive stimulation of curarized preparations produced a similar effect.

D-Tubocurarine block. In two preparations, transmission was blocked with D-tubocurarine and epps were recorded with an intracellular electrode before and after the addition of bretylium, 0·49 mM. The extent of the changes varied from cell to cell. The amplitude of the epps was reduced to 0·5 of the control or less and the duration of the epp at half amplitude was increased by up to 1·5 times.

Magnesium block. In three experiments neuro-muscular transmission was blocked in saline containing 16 mM Mg^{++}. Records were made of mepps and about 200 epps before and after the addition of bretylium. The mean quantal content of the epp was calculated by analysis of the variance of the amplitude of the epps (Martin, 1966). After bretylium, the amplitude of epps and mepps was reduced and their time courses prolonged, but there was no significant change in the mean

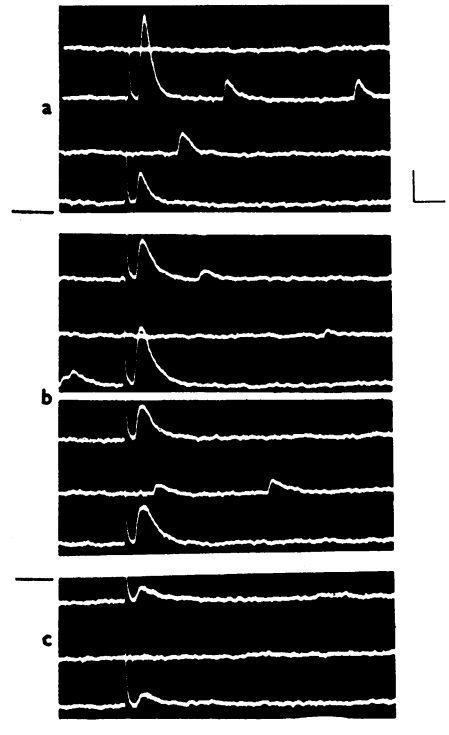

FIG. 2. Rat diaphragm, room temperature, intracellular recording from one cell with membrane potential 65 mV. Records show mepps and epps elicited in the presence of Mg^{++} 16 mM. These are part of several records taken during an experiment to determine the mean quantal content of the epp. (a) Control conditions, mean epp 1·92 mV, mean mepp 0·49 mV; (b) 10 min after bretylium, 0·12 mM, mean epp 1·11 mV, mean mepp 0·26 mV; (c) 10 min after bretylium, 0·36 mM, mean epp 0·34 mV. Calibrations: 1 mV and 10 ms.

quantal content of the epp. In the experiment shown in Fig. 2, the mean quantal content of the epp under control conditions was 4·15. Following the addition of bretylium, 0·12 mM, the mean quantal content was 4·18, epps and mepps had a duration at half amplitude which was 1·29 of the control, the mean amplitude of mepps had decreased to 0·53 of control and that of epps to 0·57. After adding bretylium, 0·36 mM, mepps were very small. The mean quantal content of the epp was 5·2, but this figure is unlikely to be accurate because it was difficult to measure accurately the epps which were reduced to 0·17 of the control amplitude.

Effect of bretylium on the electrical properties of muscle cells

It is possible that the changes in epps and mepps could have been due to alteration of the membrane characteristics of the endplate region. Two microelectrodes were inserted close together near the endplate region of a muscle cell. A hyperpolarizing current of $0{\cdot}5 \times 10^{-7}$ A was passed through one microelectrode and the membrane potential was recorded through the other. Following the addition of bretylium, 0·49 mM, the electrotonic potential was unchanged, the input resistance remaining at 0·5 MΩ.

The effect of bretylium on the membrane potential was investigated by measuring the resting potentials of a population of cells before and after the addition of the drug. The mean resting potential of 33 cells was 58·7 mV (S.E. 1·36 mV) and after adding bretylium, 0·49 mM, the mean potential of a further twenty-eight cells was 63·5 mV (S.E. 1·34 mV). After washing out the drug, the mean resting potential of another eighteen cells was 58·1 mV (S.E. 2·1 mV).

It can be concluded that after adding bretylium characteristics of the membrane at rest are unchanged. Therefore some other explanation must be sought for the effect of the drug on mepps and epps.

Effect of bretylium on ACh and carbachol potentials

In these experiments a microelectrode was inserted into the endplate region of muscle cells to record the membrane potential. A micropipette filled with ACh or Carb was placed outside the same cell and its position was adjusted until the potential recorded across the cell membrane when the drug was ejected electrophoretically had a short time course. The pulse of current ejecting the drug was arranged to give a response of 1–2 mV in amplitude, and a backing current was applied to restrain diffusion of the drug from the pipette; the intensity of the backing current was adjusted until it was just large enough to prevent depolarization of the cell in the absence of ejecting pulses. The ejecting pulses were applied at a frequency of 0·1 or 0·2 Hz. Bretylium was added to the bath as a concentrated solution and a period of about 2 min was needed for mixing to be complete.

The first experiments were carried out at room temperature and it was found that bretylium had a biphasic effect on the response of the cell to electrophoretically applied ACh. The early changes occurred 5–15 s after the addition of bretylium to the bath and consisted of an increase of the amplitude of the ACh potential to about 1·1 of the control and of a similar increase in its duration at half amplitude (Fig. 3a,b). Thereafter the amplitude fell whilst the time course was further prolonged to 1·5–2 times the control (Fig. 3,c,d,e). This prolongation of the time course suggested that bretylium prolonged the duration of action of the agonist, perhaps by an anti-

cholinesterase action. Similar experiments were carried out in which the agonist applied electrophoretically to the endplate was Carb ; in these experiments the early phase of potentiation of amplitude was not seen, only depression ; nor was there any change in the time course.

In a second series of experiments ACh and Carb potentials were elicited in the same cell and the effect of bretylium on these potentials was investigated, the experiment being done at 36° C. Carb and ACh were ejected in turn from a double-barrelled micropipette, and bretylium was added to the bath as before. The results of one such experiment are shown in Fig. 4. Initially, the amplitudes of the ACh and Carb potentials were similar, but the duration at half amplitude of the Carb potential was 2·27 times that of the ACh potential. After the addition of bretylium, 0·065 mM, the amplitude of the Carb potential fell to 0·098 of its control, whereas the ACh potential fell to 0·28 of its control amplitude and its duration at half amplitude increased to 1·21 of its control. The concentration of bretylium needed to depress the ACh and Carb potentials was much less at 36° C than at room temperature. After the bretylium was washed out, the amplitudes of the potentials returned towards the control values, but the ACh potential remained with a time course twice as long as the control. The observation that there was no prolongation of the effect of the stable cholinester, carbachol, eliminates the possibility that the prolongation of the time course of ACh action is due to prolongation of events subsequent to agonist-receptor combination and supports the hypothesis of an anti-cholinesterase action of bretylium.

Attempts were made to construct dose-response curves for the reduction of the amplitude of ACh potentials. The intensity of the ejecting current was varied and

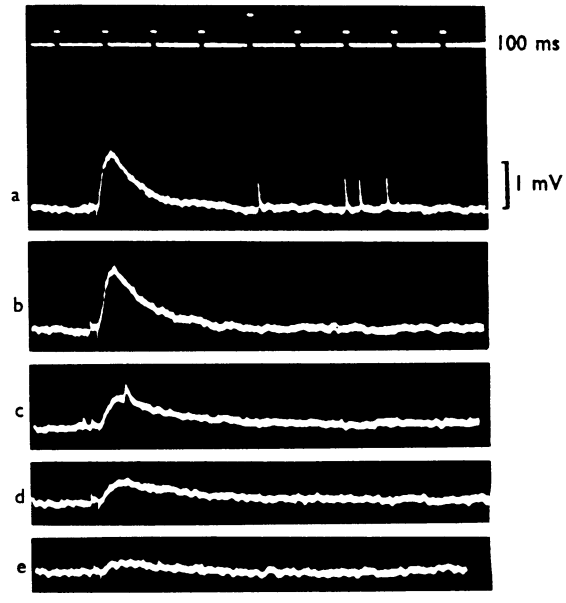

FIG. 3. Rat diaphragm, room temperature, intracellular recording from one cell with membrane potential 58 mV. Records show mepps and the potential following the microelectrophoretic application of ACh by ejecting pulse of current 10 ms in duration. (a) ACh potential and mepps under control conditions. Time 100 ms, vertical calibration 1 mV ; (b) ACh potential 5 s after the addition of bretylium to give a final concentration of 0·49 mM. Note increased amplitude and time course ; (c) 30 s after the addition of bretylium ; (d) 1 min after the addition of bretylium ; (e) 3 min after the addition of bretylium when mixing was complete.

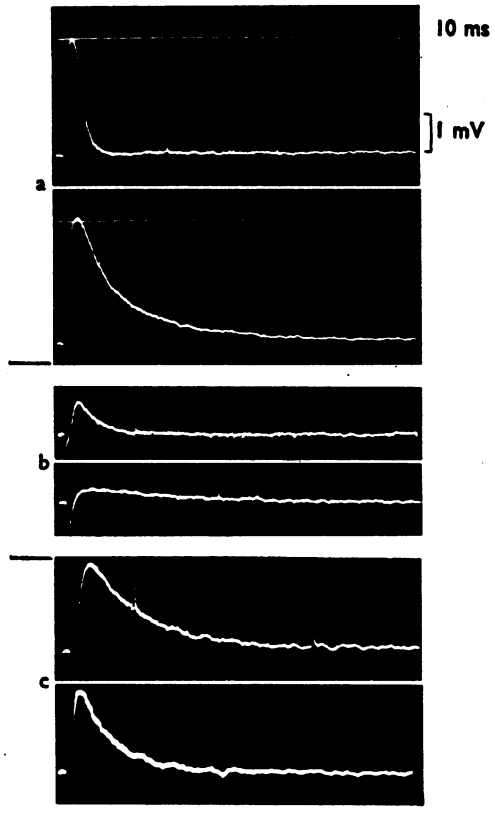

FIG. 4. Rat diaphragm, 36° C. Intracellular recording from one cell with membrane potential 52 mV. Records show the potentials following the microelectrophoretic application of ACh or of Carb from one or other of the barrels of a double-barrelled micropipette by an ejecting current pulse of 0·75 ms duration. Pulses were applied alternately to the two barrels at a frequency of 0·1 Hz. Duration of the sweep was 500 ms. (a) Upper record shows ACh potential, control conditions. Lower record shows Carb potential elicited 10 s later; (b) upper record shows ACh potential 140 s after the addition of bretylium 0·065 mM. Lower record shows Carb potential recorded 10 s later; (c) upper record shows Carb potential 3 min after washing. Lower record shows ACh potential 10 s later. Time 10 ms, vertical calibration 1 mV.

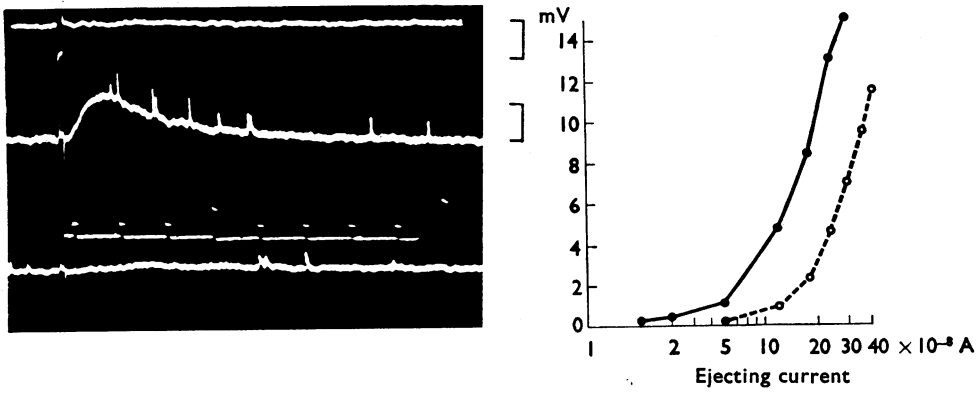

FIG. 5. Rat diaphragm, room temperature, intracellular recording from cell with membrane potential 64 mV. Left: records show mepps and the potential following electrophoretic ejection of ACh by 6×10^{-8}A current pulse of 10 ms duration. Top trace, current monitor, calibration 10^{-7}A; second trace, ACh potential and mepps under control conditions; third trace, 100 ms time mark, calibration 1 mV; fourth trace, ACh potential and mepps recorded 5 min after bretylium, 0·24 mM. Right: same experiment, graph showing the relation between the amplitude of the ACh potential (ordinate) and the intensity of the ejecting current pulses of 10 ms duration (abscissa) under control conditions (●——●) and 5 min after the addition of bretylium 0·24 mM (○- - -○).

a series of potentials of different sizes recorded before and after the application of bretylium. Satisfactory results were scarce because the cell often depolarized during the rather protracted experiments and Fig. 5 shows the results from the only experiment in which this did not happen.

In all experiments in which ACh or Carb was applied by microelectrophoresis, mepps were recorded to ensure that the microelectrode had been inserted near the endplate, and these mepps were reduced in amplitude after the addition of bretylium. Attempts were made to determine whether mepps and ACh potentials were diminished to the same extent. The amplitude of the ACh potential was adjusted to be similar to that of the mepps, and it was found that mepps and ACh potentials were similarly diminished after the addition of bretylium (Figs. 3, 5). A strict correspondence was not expected because it was evident from the very different time courses of mepps and ACh potentials that the presentation of ACh to the endplate from nerve terminals is different from the presentation from an ACh filled micropipette.

It can be concluded that bretylium exerts an anti-ACh and anti-Carb action at the endplate. This effect is temperature dependent and is exerted to a similar extent on ACh released spontaneously from nerve terminals and on that applied electrophoretically from a micropipette.

The duration of the ACh potential, but not that of the Carb potential was prolonged. This suggests that ACh might persist longer at the synapse after the addition of bretylium and that such an action might account for the widening of epps and mepps. Such an action might be produced after the inhibition of acetylcholinesterase.

It seems that the facilitatory actions of bretylium on ACh potentials appear first when the bretylium is applied to the diaphragm, and persist after the bretylium is washed out.

Effect of bretylium on acetylcholinesterase

Acetylcholinesterase activity was determined by a titrimetric method. The enzyme was prepared by washing human erythrocytes five times with distilled water and once with saline and finally suspending the packed ghosts in nine volumes of saline. The saline used had a composition similar to that given in methods, but lacked $NaHCO_3$, NaH_2PO_4, dextrose and the gas mixture. The Michaelis constant of the preparation of enzyme for the hydrolysis of ACh at pH 7·4 and 37° C was determined on four occasions by measuring the rate of hydrolysis of ACh in a range of concentrations. The maximum rate of hydrolysis was achieved with ACh concentrations of 3–10 mM ; at higher concentrations the rate of hydrolysis was less.

For each substrate concentration 0·5 ml of the enzyme preparation was diluted with saline to a final volume of 6 ml. Each reaction vessel was preincubated at 37° C for 10 minutes. The pH was adjusted to 7·4 and the reaction was started by the addition of a concentrated solution of substrate from a microsyringe to give a final concentration of 0·05–2 mM. The Radiometer titrator recorded the time course of the addition of 0·005 N NaOH added to maintain the pH at 7·4. The slope of this record was used as a measure of the rate of hydrolysis of the substrate. Two determinations were made at each substrate concentration, and corrections were made for the spontaneous hydrolysis of ACh and for spontaneous acid production.

The results were expressed as a Lineweaver-Burk plot and the regression line was calculated by the method of least squares. The mean Km determined from the four individual experiments was 0·13 mM (S.E. 0·009 mM). In two further experiments the same procedure was followed but the reaction mixture contained bretylium which had been preincubated with the enzyme for 10 minutes. The results of these experiments were expressed as Lineweaver-Burk plots. The mean value of the Ki of bretylium was 0·053 mM (S.E. 0·009 mM) and was determined from the intercepts on the ordinates of these plots and from the mean value of Km.

The results of these experiments are shown graphically in Fig. 6. Examination of these graphs shows that in the presence of bretylium there was a shift of the intercept on the abscissa towards the origin, which indicates a competitive inhibition of the enzyme. Because different preparations of enzyme were used, the maximum velocity was different on each occasion and this accounts for the scatter of the intercepts on the ordinate. Although a noncompetitive inhibition cannot be excluded due to this scatter of values of *V*max, any noncompetitive inhibition could only be small in comparison with the competitive inhibition exerted by bretylium.

Decurarizing effect of bretylium

Experiments were made on six preparations in which neuromuscular transmission had been blocked at 36° C with D-tubocurarine. An insulated wire electrode was placed extracellularly at the endplate region to record the field potentials set up by the nerve action current and by the endplate current. The phrenic nerve was stimu-

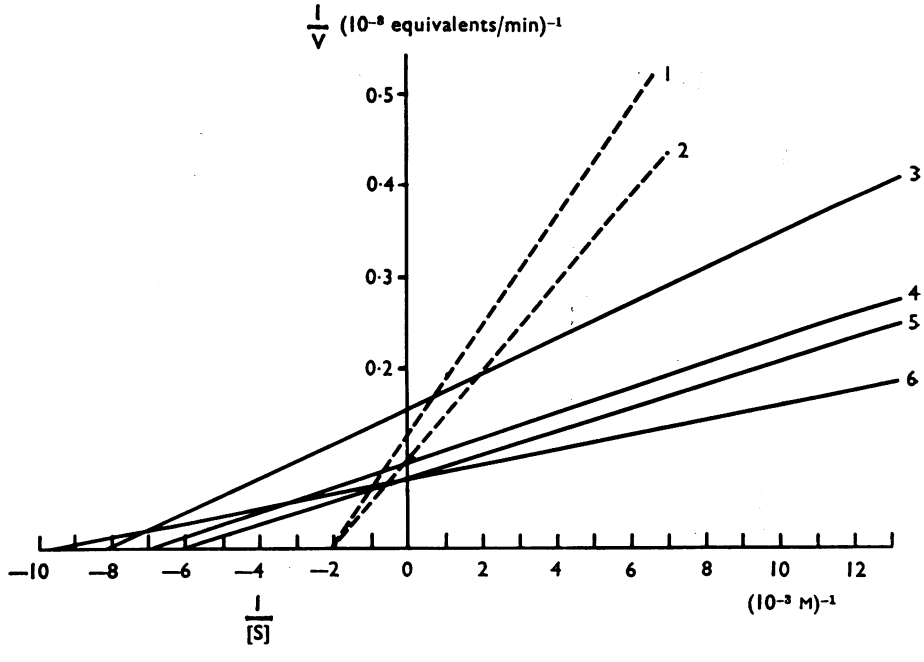

FIG. 6. Relationship between the velocity of hydrolysis of ACh in the presence of a preparation of erythrocyte acetylcholinesterase plotted on Lineweaver-Burk coordinates. Lines were calculated by the method of least squares from the experimental data obtained as described in the text. Lines 3, 4, 5, and 6 are from experiments with different batches of enzyme. Lines 1 and 2 are from experiments in which bretylium was present in concentrations of 0·16 mM and 0·12 mM respectively.

lated at a frequency of 0·1 Hz and control responses were recorded. Then bretylium tosylate was added and allowed to equilibrate for 10 minutes. After the addition of bretylium in concentrations lower than 0·25 mM, there were increases in the amplitude, area and duration of the extracellularly recorded endplate potentials; the amplitude was increased to 1·1, the area to 1·3, and the duration at half amplitude to 1·47 of the appropriate control values.

If the concentration of bretylium was less than 0·25 mM, spike potentials were recorded and the muscle was seen to twitch. When concentrations of bretylium higher than 0·25 mM were used, the duration of the endplate potential was further increased up to 2·4 of the control, and the amplitude and area of the potential were reduced and no spikes were seen. These results are depicted in Fig. 7 which shows the effects of various concentrations of bretylium on the extracellularly recorded potentials at the endplate. With a wire electrode located at the endplate region, the maximum intensity and duration of the synaptic currents and the total transfer of charge would be related to the amplitude, time course and area of the extracellularly recorded endplate potential. It may be that the amount of charge transferred is the best estimator of acetylcholine action and that the increase in the area of the endplate potential indicates an increase in the transmitter action. Similarly the decrease in area of the endplate potential may be attributed to the anti-ACh actions of bretylium. It would appear that at low concentrations of bretylium there is a predominantly facilitatory influence on transmission due to an anti-cholinesterase action, but that at higher concentrations, the anti-ACh effect overwhelms the facilitatory influence, which, however is still manifest in the prolongation of the duration of the endplate potential.

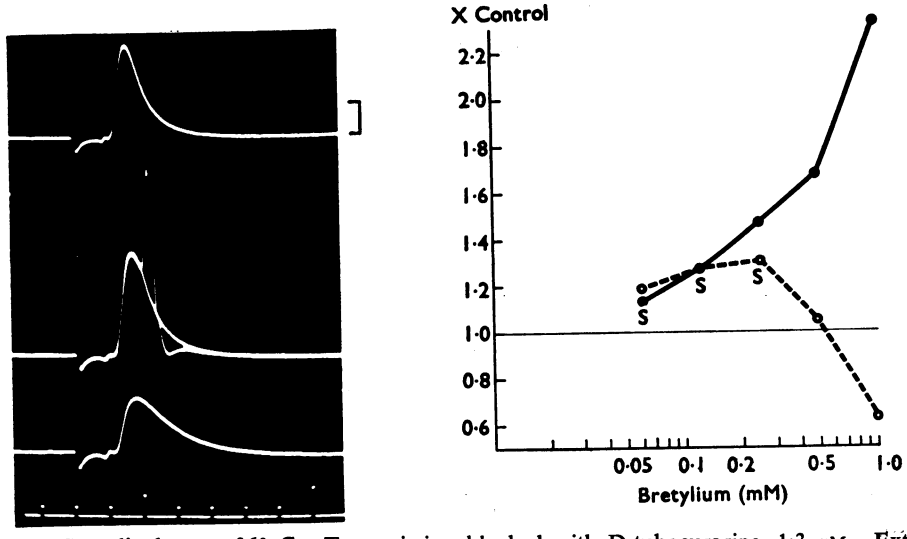

FIG. 7. Rat diaphragm, 36° C. Transmission blocked with D-tubocurarine, 1·3 μM. Extracellular electrode placed at the endplate region to record the field potential set up by stimulation of the phrenic nerve at 0·1 Hz. Left: top trace, record of the presynaptic spike and extracellular endplate potential under control conditions. Calibration 0·5 mV; second trace, two superimposed records made 5 min after the addition of bretylium, 0·06 mM; third trace, record made 5 min after bretylium 0·49 mM; fourth trace, 1 ms pulses. Right: same experiment, graph showing the relation between the concentration of bretylium (abscissa) and the duration of the endplate potential (●——●) and the area of the endplate potential (○- - -○) both plotted on the ordinate relative to the control response. For further details see text. Responses in which spikes were generated are marked S.

Experiments on frog rectus muscle

Previous workers on the neuromuscular blocking effect of bretylium reported that the drug in concentrations ranging from 1·45 μM to 2·43 mM enhanced but did not depress the contractile response of the frog rectus muscle to ACh (Dixit *et al.*, 1961 ; Gokhale, Gulati, Kelkar & Joshi, 1963). In view of the evidence given above that bretylium exerts an anti-ACh action at the rat neuromuscular synapse, it was decided to reinvestigate its action on frog rectus muscle.

Records were obtained of the contractile response to ACh, and a dose of this agonist was selected which gave responses which were about half maximal. Bretylium, 0·25 mM, was added to the bath 15 min before the next dose of ACh (Fig. 8). The response to this dose of ACh was larger than the control contracture, the mean value of this first response in the presence of bretylium being 1·87 of the control (S.E. 0·16, five experiments). Subsequent responses to ACh were progressively smaller and fell to 0·29 of the control (S.E. 0·06). After treating the rectus with eserine, the response to ACh was enhanced and therefore the dose of agonist was reduced to $0{\cdot}15 \times 10^{-6}$ which gave responses of about the same size as the original control. These responses to ACh in the presence of eserine were not enhanced after the addition of bretylium, but depression of the response was immediate and complete. The response of the eserinized rectus to $1{\cdot}5 \times 10^{-6}$ ACh in the presence of bretylium was about the same as the response to $0{\cdot}15 \times 10^{-6}$ ACh in the absence of the drug. It was concluded that after bretylium the response of the rectus to ACh was enhanced only if there had been no inactivation of cholinesterase.

In similar experiments in which the contractor response of the rectus to carbachol was recorded, after the addition of bretylium these responses were reduced to about 0·04 of the control.

It was concluded that the two actions of bretylium which are described above, an anticholinesterase action and an anti-ACh action are also exerted on the frog

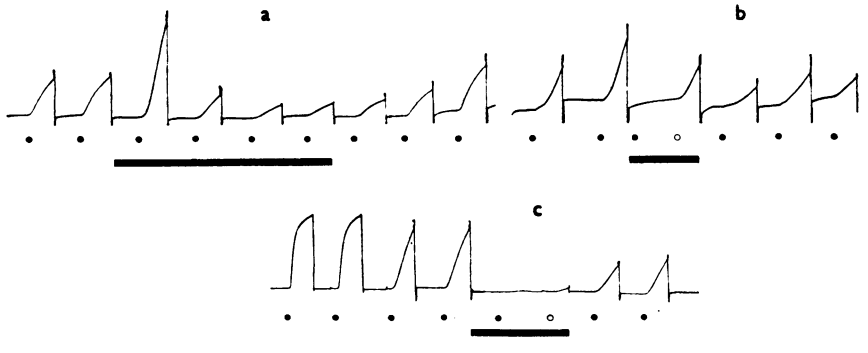

FIG. 8. Frog rectus muscle. Agonists allowed to act for 2·5 min then washed out ; rest period 15 minutes. Drugs were added at the beginning of the rest period and allowed to act for 15 min before the next dose of agonist. (a) Contractor responses (●) to ACh, 3×10^{-6}M (approximately the ED50), alone and in the presence of bretylium, 0·25 mM, indicated by the horizontal line in this and other tracings. This tracing shows the facilitation and depression of the response. (b) Contractor responses (●) to ACh, $0{\cdot}15 \times 10^{-6}$M, when eserine, 6×10^{-6}M, was present throughout. At (○) the dose of ACh was $1{\cdot}5 \times 10^{-6}$M. The tracing shows responses before and after the addition of bretylium. (c) Contractor responses (●) to carbachol, $7{\cdot}5 \times 10^{-6}$M, and (○) to carbachol, 15×10^{-6}M. The tracing shows the depressant effect of bretylium on the responses.

rectus preparation. It is clear that the facilitatory effects were shown before the depressant effects when the drug was added to the bath. If the response of the rectus to ACh is tested shortly after the addition of bretylium, only facilitation might be seen despite the fact that massive doses of the drug are added to the bath. Previous workers tested the response to ACh 0·5–2 min after adding bretylium to the preparation and found no depression. In the experiment shown in Fig. 8, the depressant effect of bretylium took 45 min to reach a steady state. It may be that the long latency of onset of the depressant effect of bretylium is a likely cause of the discrepancies between our results and those of previous workers.

Effect of bretylium on the spike potential of the diaphragm

Green & Hughes (1966) found that bretylium, 0·61 mM, had no effect on the contractor response of two diaphragms to stimulation of the phrenic nerve. These results have been confirmed in experiments done at 36° C in which the field potential was recorded with a wire electrode placed extracellularly at the endplate region. This field potential is due to extracellular currents arising from the impulse in the nerve terminals, the endplate potential and the action potential of muscle cells. After making records of the control responses to stimulation at 0·1 Hz, bretylium, 0·49 mM, was added and allowed to equilibrate for 10 minutes. In some diaphragms

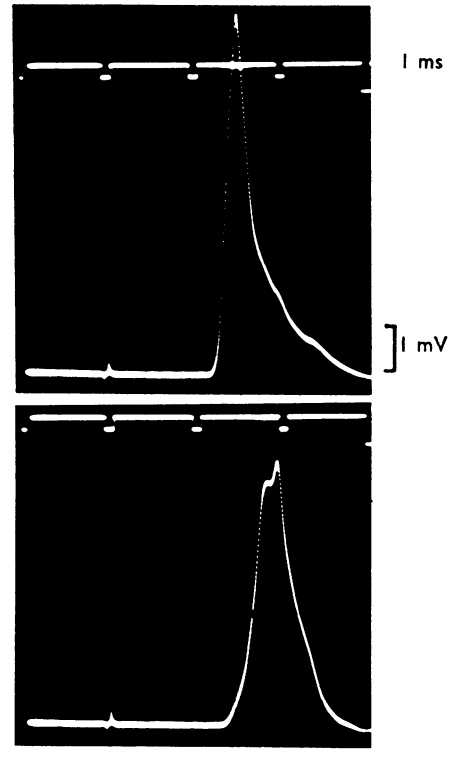

FIG. 9. Rat diaphragm, 36° C. Extracellular electrode placed at the endplate region to record the field potential set up after stimulation of the phrenic nerve. Top: the muscle spike recorded under control conditions. Lower: the response recorded 7 min after bretylium 0·49 mM. Note the decreased amplitude and increased latency of the spike. The area of this response was 0·89 of the control.

there was no change in the extracellularly recorded spike potential. In most preparations, the spike potential was of longer duration but of lower amplitude and the area was reduced to 0·86 of the control. The latency of the potential measured from the stimulus artifact to the foot of the spike was increased to 1·08 of the control (Fig. 9). Perhaps this increased latency is due to an increase in the conduction time along the nerve, for in experiments in which the presynaptic spike was recorded (Fig. 7), the latency of the negative peak of the presynaptic spike was increased to 1·08 after the addition of bretylium. Bretylium also has an effect on the threshold of the phrenic nerve fibres to electrical stimulation, for in an experiment in which the stimulus to the phrenic nerve was just maximal, after the addition of bretylium, it became submaximal. Bretylium has local anaesthetic properties (Boura & Green, 1965), and the increase in conduction time and in the threshold of the phrenic nerve fibres could be manifestations of this property.

It was concluded that bretylium in a concentration of 0·49 mM had some depressant effect on the generation of action potentials in the population of muscle cells in the rat diaphragm. Perhaps only a small effect is seen because any neuromuscular blocking drug must reduce the transmitter action to about 0·25 before transmission begins to fail (Paton & Waud, 1967), and the combined anti-ACh and anticholinesterase actions of bretylium erode the margin of safety of transmission to this extent in only 14% of junctions.

Discussion

It is clear that bretylium can produce neuromuscular block by two types of action (Dixit *et al.*, 1961 ; Green & Hughes, 1966). One action is exerted by concentrations of bretylium higher than 0·6 mM and the block comes on in the absence of conditioning stimulation of the nerve, whilst the second type of action requires a period of nerve stimulation for its development and can be obtained with concentrations of 0·25 mM. The experiments described in this paper are concerned with establishing the actions of bretylium in the absence of conditioning trains of stimuli to the nerve.

It would seem that the greater part of the neuromuscular blocking effect of bretylium in high doses is due to its anti-ACh action. There can be no doubt that the drug has a considerable anti-ACh action because epps, mepps, ACh and Carb potentials, extracellular endplate potentials and the contractile response of the frog rectus muscle to ACh and to Carb can all be diminished by bretylium. There is evidence that epps, mepps, and ACh potentials are all similarly reduced which suggests that a single antagonistic action of bretylium may be responsible. It seems that bretylium is a competitive inhibitor of ACh at the endplate.

At the same time, bretylium is capable of facilitating the responses to ACh. In both diaphragm and rectus muscle the response to the application of Carb was reduced to a greater extent than the response to the application of ACh. If the concentration of bretylium is not too high the response to ACh may even be enhanced. It is likely that this facilitation is due to a persistent action of ACh, for the duration of epps, mepps, ACh potentials and extracellular endplate potentials was increased, and that this effect is due to an antiacetylcholinesterase action of bretylium exerted at the neuromuscular junction because there is no facilitation of the response to carbachol. Furthermore, bretylium is a competitive inhibitor of

erythrocyte acetylcholinesterase, with a Ki value of 0·053 mM. In a concentration of 0·05 mM, bretylium was found partly to unblock the curarized diaphragm (Fig. 7), and if the cholinesterase of the diaphragm is affected by bretylium to the same extent as erythrocyte cholinesterase, the anticholinesterase action of the drug would be sufficient to account for its decurarizing effect.

If bretylium were to inactivate a considerable proportion of the acetylcholinesterase at the endplate, as well as exert an anti-ACh effect great enough to cause neuromuscular block, it might be expected that this block would not be reversed by anticholinesterase drugs. Such an explanation might account for the fact that the neuromuscular block produced in the isolated rat diaphragm by bretylium 0·24–0·79 mM was not reversed by eserine (Dixit *et al.*, 1961), or by neostigmine (Boura & Green, 1959).

Thus, there is simultaneous facilitation and depression of neuromuscular transmission and it seems that with concentrations of about 0·5 mM bretylium these effects offset one another giving the deceptive result that the drug appears to have little or no effect.

In experiments on the rectus and on the diaphragm the responses to ACh were potentiated soon after the addition of bretylium and also after it had been washed out. These results suggest that the facilitatory actions of bretylium are manifest at low concentrations of the drug and the depressant action is only seen at higher concentrations. This interpretation is supported by the fact that the decurarizing effect of bretylium was seen only at low concentrations.

There is no evidence that bretylium, under the conditions of these experiments, has any presynaptic actions. The constancy of the frequency of mepps suggests that there has been no change in the polarization of the nerve terminals. Nor is it likely that there is any alteration in the action potential—secretion coupling mechanism because the mean quantal content of the epp was unaltered, although it must be remembered that these experiments were done in high Mg^{++} where the quantal content is already low. According to the quantum theory of transmitter release, the quantal content of the epp is the product of the number of quanta in the available store and the probability of release of each quantum following a nerve impulse. The run down of the epp with repetitive stimulation is considered to be due to the partial exhaustion of the available store by the preceding impulses in the train (for references see Hubbard, Llinas & Quastel, 1969). The rundown of the epp in cells where transmission was blocked in bretylium 2·4 mM was similar to that found in curarized preparations and it may be concluded that bretylium has no effect on the size of the presynaptic store or on the probability of release of quanta.

It is also unlikely that bretylium blocks the conduction of the impulse in the phrenic nerve terminals, for in experiments on junctions blocked with bretylium 2·4 mM an epp was recorded intracellularly each time the nerve was excited with supramaximal stimuli. Although bretylium exerts a local anaesthetic action, it appears that if an impulse has been generated by a supramaximal stimulus, the safety margin of propagation along the nerve is sufficient to ensure its arrival at the terminals. However, conduction is slowed to a slight extent and the threshold of the nerve is increased. It is possible that an elevation of the threshold of the muscle membrane might contribute towards the production of block of transmission.

To conclude, the blocking effect of bretylium on neuromuscular transmission is due to its anti-ACh action, but this action is offset by a simultaneous inhibition of

acetylcholinesterase. There is no evidence that presynaptic actions are involved in the block which comes on in the absence of nerve stimulation: the actions of bretylium involved in the stimulation dependent block will be reported at a later date.

To return to the initial question of the mode of action of bretylium at adrenergic synapses, there are several hypotheses of the mode of action of adrenergic neurone blocking agents. Boura & Green (1965) suggested that bretylium had a weak local anaesthetic activity which was exerted to a greater extent in adrenergic fibres because of the preferential uptake of the drug by these cells. Burn & Rand (1965) have suggested that drugs which have anti-ACh actions and which also block adrenergic neuro-effector transmission might act on some cholinergic link. Similarly, drugs which facilitate adrenergic transmission and which have anticholinesterase properties might be acting at this cholinergic link in adrenergic transmission. The experimental results which are described in this paper shed no further light on the mode of action of bretylium at adrenergic synapses.

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REFERENCES

BOURA, A. L. A. & GREEN, A. F. (1959). The actions of bretylium: adrenergic neurone blocking and other effects. *Br. J. Pharmac. Chemother.*, **14**, 536–548.

BOURA, A. L. A. & GREEN, A. F. (1965). Adrenergic neurone blocking agents. *Ann. Rev. Pharmac.*, **5**, 183–212.

BURN, J. H. & RAND, M. J. (1965). Acetylcholine in adrenergic transmission. *Ann. Rev. Pharmac.*, **5**, 163–182.

CURTIS, D. R. (1964). Microelectrophoresis, In: *Physical Techniques in Biological Research*, ed. Nastuk, W. L., Vol. **5**, Electrophysiological Methods part A. pp. 144–192. London: Academic Press.

DIXIT, B. N., GULATI, O. D. & GOKHALE, S. D. (1961). Action of bretylium and guanethidine at the neuromuscular junction. *Br. J. Pharmac. Chemother.*, **17**, 372–379.

GREEN, A. F. & HUGHES, R. (1966). Effects of adrenergic neurone blocking agents on voluntary muscle stimulated at different frequencies. *Br. J. Pharmac. Chemother.*, **27**, 164–176.

GOKHALE, S. D., GULATI, O. D., KELKAR, V. V. & JOSHI, N. Y. (1963). Effect of bretylium and guanethidine on some cholinergic effectors. *Arch. int. Pharmacodyn. Ther.*, **145**, 243–253.

HUBBARD, J. I., LLINAS, R. & QUASTEL, D. M. J. (1969). *Electrophysiological analysis of synaptic transmission.* London: Edward Arnold (Publishers) Ltd.

MACINTOSH, F. C. & PERRY, W. L. M. (1950). Biological estimation of acetylcholine, in: *Methods in Medical Research*, ed. Gerard, R. W., Vol. **3**, pp. 78–92. Chicago: The Year Book Publishers Inc.

MARTIN, A. R. (1966). Quantal nature of synaptic transmission. *Physiol. Rev.*, **46**, 51–66.

PATON, W. D. M. & WAUD, D. R. (1967). The margin of safety of neuromuscular transmission. *J. Physiol., Lond.*, **191**, 59–90.

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