EFFECT OF ACETYLCHOLINE IN THE ELECTROPLAX OF ELECTRIC EEL*

by

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INTRODUCTION

Acetylcholine reduces the transmembrane potential measured across the resting innervated membrane of the electroplax of electric eel[1]. It inhibits also the action potential, until it eventually disappears[1]. This effect is of considerable interest since acetylcholine, like other quaternary ammonium compounds, has little or no pharmacological action on the membrane of cells that present propagated action potentials, as axon[2,3] or striated muscle[4,5,6]. The innervated membrane of the electroplaque is a rather complex structure by the fact that a great number of small post-synaptic areas are scattered in a membrane that for brevity will be called non-synaptic membrane. It could be appropriately compared with the membrane of striated muscle if multiple end plates were distributed all over the fiber surface. The postsynaptic region of the electroplaque has similar physiological characteristics as the end plate of striated muscle[7]. Corresponding similarities exist in the behavior of the membranes at the non-synaptic areas of electroplax and striated muscle[7]. It is obvious, therefore, that the interpretation of the action of acetylcholine upon the electroplaque becomes more meaningful after its site of action is determined.

This paper deals with an attempt to localize the site of action of acetylcholine and report some related observations on its pharmacological effect. The experiments were performed with a preparation recently developed[8] that allows the simultaneous excitation of the whole innervated membrane of the electroplax by currents of known density. This method permits the study of the excitability of the preparation, the measurement of the membrane resistance, and the study of the membrane characteristics under different trans-membrane potentials. Also, since for this type of experiment, the electroplax must be freed of all extraneous tissue, the diffusion of chemical compounds from the solution to the cell membrane is markedly accelerated when compared with previously used preparations.

METHODS

Since the procedures employed have been described and discussed in another paper[8], they will be only briefly outlined here.

A single row of five to seven electroplax was carefully cleaned of all extraneous tissue and

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was transfixed at its ends by two silver wires. The wires were connected to the outputs of a stimulus isolation unit. The voltage drop across a 100 ohm resistance in series with the isolation unit was utilized to measure the current delivered by the latter. For example, in Figs. 6 and 7 the upper trace of each record shows the potential difference produced by the stimulating rectangular pulse across the 100 ohm resistance. The preparation may be maintained in air or in saline solution. When it was kept in air, the whole cell membrane was stimulated simultaneously and a stationary action potential was elicited. The records of Figs. 6 and 7 were obtained by this method. The area of the membrane of the cell studied was measured at the end of the experiment[8] and the density of the stimulating current was calculated.

The electroplax could also be stimulated by a pair of pin point electrodes straddling the cell. In this case a discrete region was excited and the elicited spike propagated along the cell membrane. All the observations made with the preparation immersed in the saline solution (Figs. 1 to 5) were obtained with this type of stimulus.

It has been previously shown[7] that the electroplax are directly stimulated by a cathodic pulse applied to the innervated membrane. The nerve terminals are excited when a stimulus of the opposite polarity is used and it is possible to elicit postsynaptic potentials.

The electrical potentials of the electroplax were recorded by means of two micropipettes filled with 3 *M* KCl, placed as near as possible to the innervated membrane, one inside the cell, the other outside.

Denervated electroplax were obtained by destruction of the spinal cord[9] 2 or more months before their use.

RESULTS

Effect of acetylcholine upon the resting and action potentials

As stated in METHODS, the preparation may be maintained immersed in the saline solution or in air during the electrical recordings. Due to purely physical reasons discussed in another paper[8], the duration and magnitude of the action potential are reduced when the first procedure is used. Despite this inconvenience, this method is more appropriate to study the effect of a drug when records must be obtained every two or three minutes. However, it might be stated that the action of any drug is qualitatively the same regardless of the method used.

The following results are based on experiments carried out with concentrations of acetylcholine varying between 0.2 and 20 μg/ml. The saline solution contained in addition eserine (5 to 10 μg/ml).

Fig. 1 illustrates a typical experiment. The ordinates at left indicate the magnitude in mV of the resting potential (closed circles) and action potential (open circles).

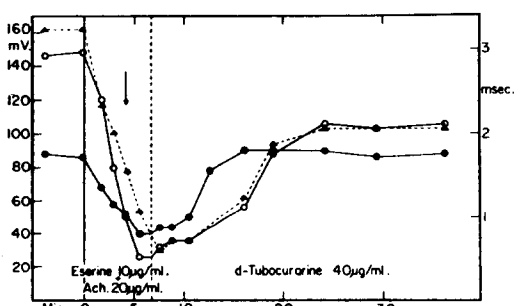

Fig. 1. Effect of acetylcholine on the resting and action potentials of the electroplax (Date: 4/30/55). Normal electroplax. The ordinates at left indicate the magnitude of the action potential (○) and resting potential (●) in mV. The ordinates at right indicate the duration of the action potential (▲) in msec. Abscissae: time in min. A solution containing acetylcholine (20 microg/ml) and eserine (10 microg/ml) was added at time zero (full vertical line). The broken vertical line shows when the solution of acetylcholine was changed to a solution of *d*-tubocarine (40 microg/ml). The arrow indicates when the action potential ceased to be propagated.

The ordinates at right indicate the duration of the action potential (closed triangles) in msec. At time zero acetylcholine (20 μg/ml) and eserine (10 μg/ml) were added to the bath. A significant decrease of the magnitude of the spike and resting potential is noticeable 1.5 min after the addition of the compounds. The decrease of the resting

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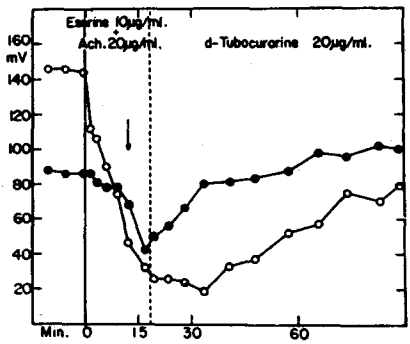

Fig. 2. Effect of acetylcholine on the resting and action potentials of the electroplax (Date: 5/2/55). Normal electroplax. The ordinates at left and the abscissae have the same significance as in Fig. 1. A solution of acetylcholine (20 µg/ml) and eserine (10 µg/ml) was added at time zero; it was changed to a solution of *d*-tubocurarine (20 µg/ml) as indicated by the vertical broken line. The arrow shows when the action potential ceased to be propagated.

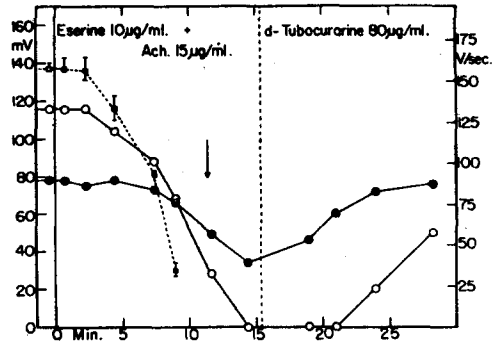

Fig. 3. Effect of acetylcholine on the resting and action potentials (Date: 7/9/55). Normal electroplax. Ordinates at left and abscissae: same as in Figs. 1 and 2. Ordinates at right: rate of rise of the action potential in volts/sec. Only the rate of rise of propagated spikes is plotted (see text). Each value (□) is the average of 9 or 10 determinations: the range of variation is indicated in each case. Acetylcholine (15 µg/ml) and eserine (10 µg/ml) were added at time zero. This solution was replaced by a solution of *d*-tubocurarine (80 µg/ml) at the time indicated by the broken vertical line. The arrow shows when the spike ceased to be propagated.

and action potentials was usually simultaneous. However, a clear inhibition of the spike was observed in three of 16 experiments at a time when the resting potential was unmodified (Figs. 2 and 3). The block of the spike by acetylcholine without depolarization may be due to the small amount of eserine added. Eserine antagonizes the depolarizing action of acetylcholine by a competitive antagonism[10, 11]. It may be possible that the concentration of eserine at the post-synaptic membrane may be much higher than the concentration of acetylcholine for a short time after the simultaneous addition of both compounds. This assumption is based upon the fact that the permittivity of biological membranes to tertiary ammonium compounds seems to be higher than to quaternary salts. The number of receptors combined with acetylcholine would slowly increase and will reach the point at which depolarization is produced only after a longer period of time. It has been experimentally demonstrated that concentrations of eserine sufficiently high to prevent the depolarization caused by a given concentration of acetylcholine, are not able to protect against the inhibition of the spike by the ester (see below). Therefore, the depolarization produced by acetylcholine may be antagonized and its effect on the action potential left untouched inspite of the ratio of concentration of eserine to acetylcholine is the same in the whole membrane. It is not surprising that this dissociation between depolarization and spike inhibition was not noticed in all the experiments performed with eserine. It is a transitory phenomenon and in many experiments the measurements were not performed with the frequency required to make this observation. The arrow in Figs. 1 to 3 indicates the time the spikes ceased to be propagated; to the right of the arrow are plotted local potentials recorded across the point stimulated.

At the broken line of Figs. 1 to 3, the saline solution containing acetylcholine was changed to one containing *d*-tubocurarine. Fig. 1 shows that 40 µg/ml of *d*-tubocurarine produce the rapid recovery of the resting potential to the normal value and

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the action potential returns to about 70% of the control amplitude, although it is not propagated. Fig. 2 illustrates an experiment in which the same amounts of acetylcholine and eserine as in Fig. 1 were used, but the concentration of *d*-tubocurarine was lower and consequently the recovery took place at a slower rate. It is important to note in Fig. 2 that the resting potential recovered to 93% of the normal magnitude without a concomitant change of the action potential. The effect of acetylcholine was more drastic in the experiment shown in Fig. 3 than in the previous illustrations, because the action potential disappeared completely. 80 μg/ml of *d*-tubocurarine caused the return of the resting potential to the normal level with only a slight recovery of the action potential. Again there is a clear dissociation between the initiation of the recovery of the resting and action potentials. A similar action was produced by other compounds that antagonize acetylcholine by competition for the same receptor. For example, procaine (100 μg/ml to 1000 μg/ml) produced the complete recovery of the resting potential without any effect on the magnitude of the inhibited activity. Obviously, the duration of the action of acetylcholine; period during which the cell has been maintained depolarized; concentration of the antagonist of acetylcholine, and other factors must play an important role in the degree of recovery of the action potential.

The action of acetylcholine is readily reversible in a great variety of tissues. On the contrary, the electroplaque does not exhibit any recovery from the effect of acetylcholine when the preparation is returned to pure saline solution. This applies to preparations treated with acetylcholine in presence of small concentration of eserine (5 to 10 μg/ml) or acetylcholine alone. The problem has been carefully investigated with similar results by means of carbamylcholine. In this case the complication arising from the hydrolysis of acetylcholine are not present. The lack of spontaneous recovery of the electroplaque after the effect of acetylcholine could be ascribed to an irreversible damage of the cell membrane. However, the experiments illustrated in Figs. 1 to 3, show that this is not the case at least within short periods, because the resting and action potentials may recover if an antagonist of acetylcholine is rapidly added. The complete recovery of the spike has not been observed, but it must be emphasized that the experiments were not designed to obtain this effect; they were only intended to demonstrate that the depolarization is reversible. In order to produce the full recovery of the spike it is probably necessary to let acetylcholine act for very short periods of time and reverse its action as fast as possible.

The duration of the spike was measured in all the experiments and in no circumstance was prolonged. On the contrary, as shown in Fig. 1, the magnitude of the action potential (open circles) and its duration (triangles) decreased simultaneously. The two effects were not always proportional. Frequently the small local activity tended to be longer than might have been expected considering its magnitude.

HODGKIN AND KATZ[12] showed with squid axon that the rate of rise of the spike is at first approximation directly proportional to the external concentration of sodium. Since then, it has been generally accepted that this rate of rise is an accurate index of the rate of change of the sodium permittivity of the membrane during the first phase of the action potential. Therefore, it was considered interesting to analyze the effects of acetylcholine on this phase of the directly elicited spike. A characteristic experiment is illustrated in Fig. 3. The ordinates at left and abscissae have the same meaning as in previous figures. The ordinates at right indicate the rate of rise of

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propagated spikes in volts per sec. Only the rate of rise of propagated spikes has been plotted in this figure. When the spike ceased to be propagated, the duration of the rising phase and its maximal slope became proportional to the stimulus strength, *i.e.* it had no definite magnitude. The rate of rise of the control action potential was about 158 V/sec. It was not modified during the first three minutes after the addition of 15 μg/ml of acetylcholine. The first noticeable action of acetylcholine was a decrease of the spike magnitude that coincided with a reduction of the rate of rise of the action potential. This latter effect was increasingly evident as the amplitude of the action potential diminished. The time constant of the recording system utilized (including capillary micropipettes) was determined at the end of each experiment. In the experiment illustrated in Fig. 3 it was about 155 μsec. If the recorded spikes are corrected by means of the "subtangent" method[13], the rate of rise of the control activity was about 178 V/sec instead of 158 V/sec (15% difference). Since the error decreased as the rate of rise diminished, the actual change was larger than indicated by Fig. 3.

The rate of rise of repeatedly elicited action potentials decreases markedly when the effect of acetylcholine is advanced. For example, four successive spikes elicited by a 1/sec stimulation at time 8 in the experiment illustrated in Fig. 3, had a rate of rise of 39, 37, 35 and 31 V/sec respectively.

Five experiments were carried out on electroplax denervated by section of the nerves five months previous to the performance of the experiments. No significant difference existed between the normal and denervated electroplaque with regard to the resting potential and spike elicited by direct stimulation[7]. When a small amount of eserine was used (10 μg/ml) the minimal concentration of acetylcholine necessary to produce the disappearance of the spike in one hour was about ten times higher in normal than in denervated cells.

In five experiments acetylcholine was used without the addition of eserine. The action of this compound has the same characteristics as described above. However, a concentration about 300 times higher was necessary to produce in normal or denervated cells (500 μg/ml and 50 μg/ml respectively) an effect similar to the action caused by a small concentration of acetylcholine when eserine was added.

Site of action of acetylcholine

It is well known that *d*-tubocurarine acts with high relative specificity at the postsynaptic region either in striated muscle or electroplax[1]. For example, a concentration of 40 μg/ml of this compound blocks the synaptic transmission of the electroplaque within 15 min but has no detectable action on the spike produced by direct stimulation within the first three hours. In order to produce an effect on the directly-elicited action potential it is necessary to use concentrations 20 or more times higher over long periods[1]. It is also known that *d*-tubocurarine antagonizes acetylcholine by competition for the same receptor[10, 11].

Experiments were performed in which the depolarizing action of acetylcholine was antagonized by small concentrations of *d*-tubocurarine. The magnitude of the postsynaptic potential was used to ascertain the effect of the drug on the postsynaptic region. Fig. 4A illustrates a characteristic experiment. The closed circles express the magnitude of the depolarization caused by acetylcholine in percentage of the maximal effect obtained. This experiment is the same as illustrated in Fig. 1 and therefore,

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a value of 100% corresponds to a decrease of the resting potential by 46 mV. The size of the postsynaptic potential just before the addition of the solution with *d*-tubocurarine is taken as the 100% amplitude of this potential. It has been previously mentioned that the postsynaptic potential, for a long period, is only slightly decreased by acetylcholine[1]. Fig. 4A shows that the recovery of the resting potential approximately parallels the inhibition of the postsynaptic potential. Also it is seen that the resting potential presents a small overshoot about the time the postsynaptic potential disappears. Fig. 4B presents a similar plot of the experiment illustrated in Fig. 2. Since the concentration of *d*-tubocurarine is lower than in Fig. 4A, the recovery of the resting potential and the inhibition of the postsynaptic potential were slower, but again the effect of *d*-tubocurarine upon both potentials was parallel.

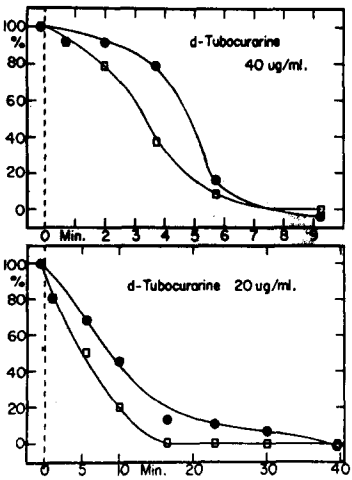

Fig. 4. Parallel action of *d*-tubocurarine on the postsynaptic potential and on the depolarization caused by acetylcholine. A and B: the ordinates express the depolarization as percentage of the maximal depolarization observed in each experiment (●). Also they indicate (□) the size of the postsynaptic potential in percentage of the reference magnitude (see text). Graph A illustrates the same experiment as shown in Fig. 1 and graph B the experiment shown in Fig. 2. In both cases the solution containing the concentration of *d*-tubocurarine indicated in the graphs was added at time zero.

A number of experiments have been performed in which a procedure opposite to the above has been followed. Compounds such as procaine, *d*-tubocurarine, tetra-ethyl ammonium and tetra-*n*-propyl ammonium, known to antagonize the depolarizing action of acetylcholine even in small concentration, have been added to the saline solution and the effect of a subsequent dose of acetylcholine has been studied. Acetylcholine in threshold concentration does not depolarize if any one of the above compounds has blocked the synaptic transmission, but the action of acetylcholine on the spike may be left unmodified, as shown in Fig. 5. In this experiment acetylcholine has been used in high concentration since no eserine was added to the saline solution. 600 μg/ml of acetylcholine block the spike in about the same time as shown in Fig. 5 whether the preparation was in pure saline solution or in a solution containing 0.02 *M* tetra-*n*-propylammonium chloride. The resting potential is not affected (Fig. 5); the

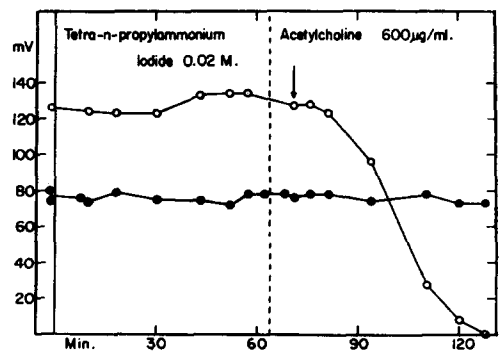

Fig. 5. Antagonism of the depolarizing action of acetylcholine by a compound that blocks with high relative specificity synaptic transmission (Date: 3/22/55) Normal electroplax. Ordinates and abscissae as in Fig. 1 to 3. A solution of 0.02 *M* tetra-*n*-propylammonium iodide was added at time zero. (The concentration of NaCl in the saline solution was correspondingly decreased.) The broken line indicates the time when acetylcholine (600 μg/ml) was added. The arrow shows when the action potential ceased to be propagated.

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same concentration of acetylcholine in the absence of tetra-*n*-propylammonium would depolarize the membrane to about 40 to 50 mV. This type of experiment shows that the action of acetylcholine on the spike may be exerted without any change of the resting potential. So far all the compounds tested that block the synaptic transmission with high relative specificity, antagonize the depolarization produced by acetylcholine. This applies to eserine, procaine, the tertiary analogue of prostigmine, *d*-tubocurarine, tetra-ethylammonium, tetra-*n*-propylammonium and tetra-*n*-butylammonium.

Effect of acetylcholine on the excitability

When the preparation described in this paper was stimulated in air, the whole innervated membrane of the electroplaque was excited simultaneously and a stationary action potential was elicited[8]. This procedure allowed precise determinations on the current sufficient to produce a threshold depolarization. The problem of whether acetylcholine increases the excitability of the non synaptic membrane of the electroplaque is important with regard to the role that acetylcholine may play in the production of the action potential (see discussion). The results obtained are inconclusive, as will be discussed below. Usually the magnitude of the threshold depolarization remained the same even when the resting and action potentials were markedly depressed. It is interesting to note that the propagation of the spike could be blocked without significant changes of the excitability of the electroplaque. An increase of the magnitude of the threshold depolarization was always evident a short time before the cell activity was completely blocked[1].

Fig. 6 illustrates the only experiment in which a clear increase of the excitability was observed. Even in this case the change is rather small. Records A, B and C are the controls of an experiment performed with a preparation denervated 4 months previously. In record A, the spike was elicited by a short stimulus. In B the membrane was depolarized by a just subthreshold rectangular pulse of 22.5 msec duration; the stimulus reached threshold in record C. The density of the current sufficient to produce the threshold depolarization (40 mV) was 1.7 mA/cm^2. Records D, E and F show the electrical potentials of the electroplaque 10 min after the addition of 500 μg/ml

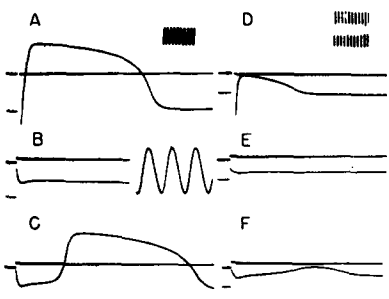

Fig. 6. Action of acetylcholine on the direct excitability of the electroplax (Date 2/14/55). Preparation denervated 4 months previously. In all records the resting level of the upper trace corresponds to the zero reference in the transmembrane potential measurements. This trace also shows the potential gradient across a 100 ohm resistance produced by the stimulating current. The lower trace shows the transmembrane potential. A: Spike elicited by a 0.1 msec pulse. The calibration at right refers to the upper trace of records A to C (100 mV). B: Just subthreshold depolarization caused by a cathodal pulse lasting 22.5 msec. At right is the time calibration for all records (1000 cycles) and the amplitude calibration for the lower traces (100 mV). C: threshold depolarization produced by a long stimulus (22.5 msec). The correct resting potential for records A to C is shown in A (82 mV). A: drift of the preamplifier falsified the measurements of the resting potential in records B and C. The records D to F were obtained immediately after the preparation being immersed 10 min in a solution of 500 μg/ml of acetylcholine. Since the preparation was denervated, this concentration produced a rapid effect. D: maximal local potential produced by a short stimulus. The calibration at right (100 mV) refers to the upper traces of records D to F. Records E and F: subthreshold and threshold stimulation caused by a pulse of 22.5 msec duration.

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of acetylcholine. The threshold current in record F was about 1.3 mA/cm² and the the electrical potentials of the electroplaque 10 min after the addition of 500 μg/ml correspondent depolarization 28 mV. This particular experiment shows the maximal change in threshold observed; however, it is difficult to know whether the increase in excitability is due to the direct effect of acetylcholine or whether it is mediated through the depolarization present.

An anodal polarization of the membrane relieved the depression or even the block of the activity caused by acetylcholine in various concentrations. This latter effect was observed only during the first few minutes of block and was not noticeable after longer periods. Under these conditions, the action of the anodal pulse was similar to the results obtained when the block had been caused by an excess of potassium. The unblocking action was present not only during the anelectrotonus, but also 20 or more msec after the electric pulse finished.

Effect of acetylcholine on the "net influx" of sodium

When the preparation in air was subjected to long rectangular pulses of appropriately directed current, the transmembrane potential of the innervated membrane could be maintained during long periods of time at any desired level[8]. Under such experimental conditions, it was possible to keep the transmembrane potential at a magnitude at which the electrochemical potential of sodium should be the same on both sides of the membrane. No net influx of sodium should take place and therefore, no action potential should be observed if the latter is produced by a net influx of sodium[14]. Whether, in this connection, the term "electrochemical potential of sodium" is correctly used will be discussed later.

Fig. 7 illustrates an experiment designed to measure the electrochemical potential of sodium. A is the control spike elicited by a 0.2 msc stimulus. The record obtained when the cell was depolarized to the point at which the electrochemical potential of sodium was apparently the same on both sides of the membrane is shown in A'. The spike does not add any potential to the voltage drop across the membrane caused by the superimposed electric field; the record obtained is a rectangular trace (A'). The resting potential in A' was falsified by a drift of the amplifier and the correct value is shown in A. Afterwards the preparation was immersed in a solution containing 50 μg/ml of acetylcholine. Since these electroplax were denervated five months previously, acetylcholine was active in small concentrations, as shown by the inhibition of the spike in record B, despite no eserine being added. The corresponding

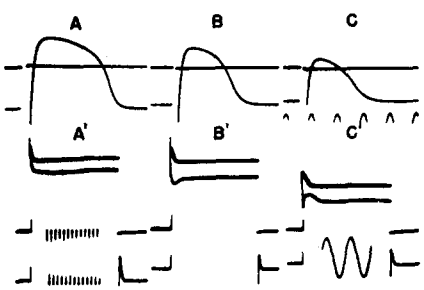

Fig. 7. Modifications produced by acetylcholine on the magnitude of the transmembrane potential sufficient to prevent a net influx of sodium (Date: 2/15/55). Electroplax denervated four months previously. A: control spike elicited by a short stimulus and A': transmembrane potential at which apparently no net influx of sodium takes place during the action potential (see text). B and B': the preparation was immersed during the last 50 mins in a solution of acetylcholine (50 μg/ml). C and C': after 70 mins in the same solution of acetylcholine. The time calibration (1000 cycles) for records A, B and C is shown in C. The time calibration (1000 cycles) for records A', B' and C' and the amplitude calibration (100 mV) for the lower traces of these records are shown in A'. The amplitude calibration (100 mV) for the upper traces is recorded in C'.

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determination of the apparent electrochemical potential of sodium is illustrated in B'. After 70 min in acetylcholine solution, the electrical potentials of the cell were as shown in records C and C'. The resting potential decreased from 82 to 63 mV and the spike magnitude diminished from 132 to 79 mV; moreover it was not propagated. The spike adds some potential to record C', consequently there was still an influx of sodium. An interpolation between record C' and the record next to C' performed with a stronger current (not included in Fig. 7) shows that the correct magnitude of the displacement of the transmembrane potential sufficient to prevent an influx of sodium ions is about 127 mV. The corresponding values in A' and B' were respectively 210 and 176 mV. When the magnitude of the corresponding resting potential is deducted from these values, it is observed that 128, 100 and 64 mV of internal positivity of the electroplaque prevented the influx of sodium in A, B and C respectively. Therefore, the decrease of the magnitude and duration of the spike coincided with a parallel decrease of the magnitude of the apparent electrochemical potential of sodium. The same results were obtained in experiments lasting less than 10 minutes, performed with higher concentrations of acetylcholine. It is improbable that any important change in the total concentration of sodium either inside or outside the cell could occur under such conditions. As it will be shown below, the resistance at rest of the non-synaptic membrane is not decreased by acetylcholine. Therefore, it is possible to suggest that the sodium permittivity of the membrane after exposure to acetylcholine at most will be not higher than in standard conditions. Since this permittivity is rather low, it is improbable that the intracellular concentration of sodium could be markedly changed within the period sufficient to observe the effects above described, *i.e.* 2 to 8 mins in some experiments.

Effect of acetylcholine on the resistance of the membrane at rest

The resistance of the innervated membrane has been determined as previously described[8]. The displacement of the transmembrane potential produced by a current of known density was measured. From these two quantities the resistance per square centimeter of membrane was derived. The results may be plotted as shown in Fig. 8A and B. The displacement of the transmembrane potential is considered negative when an anodal pulse is applied, because the inside of the cell, compared with the outside, is more negative than in resting condition. The anodal current is also considered negative. The opposite sign is used for cathodal pulses and for the changes of the transmembrane potential produced by them. The open circles in Fig. 8A and B are the result of control measurements. The arrows at right indicate the depolarization at which a spike was elicited by the cathodal pulse in each phase of the experiment. The results to the right of the arrows, *i.e.* after the cell has been excited, will not be considered in this paper. Therefore, the following description refers only to the magnitude of the resistance of the membrane at rest. As described previously[8], the resistance of the membrane at rest behaves as an ohmic resistance and there is no recognizable rectification provided no action potential is elicited.

The experiment illustrated in Fig. 8A was performed with a preparation denervated 4 months earlier. The resistance of the membrane at rest is 14.7 ohm/cm^2 (open circles). 500 μg/ml of acetylcholine in 8 min decreased the resting potential from 86 mV to 46 mV and the action potential from 156 to about 54 mV. This experiment is also illustrated in Fig. 6. Despite these important changes, the resistance of the

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membrane at rest (crosses) was not modified. Immediately afterwards, the preparation was washed repeatedly with saline solution. As stated before, under these conditions, the effect of acetylcholine is not reversible and 100 min later the resting potential was 22 mV and no activity whatsoever could be elicited. The resistance as shown by the squared in Fig. 8A was about 3 ohm/cm². A similar irreversible decrease in resistance was observed whenever the membrane was damaged by means of physical or chemical procedures.

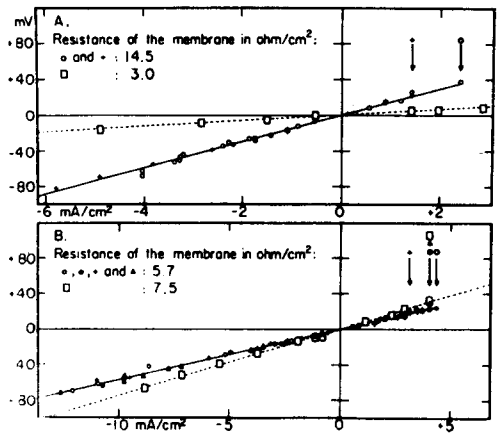

Fig. 8. Action of acetylcholine on the resistance of the membrane at rest. In graphs A and B the ordinates indicate the displacement of the transmembrane potential in mV produced by a current of known density (abscissae in mA/cm²). Negative displacements are produced by anodic shocks and positive displacements by cathodal currents (see text). The arrows show when the depolarization reached threshold: the analysis of the curves to the right of the arrows will be omitted in this paper. Graph A (Date 2/14/55): Electroplax denervated 4 months previously. Control values (o). The preparation was immersed in 500 μg/ml of acetylcholine and tested 8 mins later (+). Immediately afterwards it was washed repeatedly with saline solution and its resistance measured again 100 mins later (□). Graph B (Date 2/18/55): Electroplax denervated 4 months previously. Control values (○). The resistance of the membrane was measured at the end of 20 min of exposure to 5 μg/ml of eserine (●). Immediately afterwards acetylcholine (0.2 μg/ml) and eserine (5 μg/ml) were added and the resistance tested again 30 (+) 50 (△) and 66 (□) min later.

Fig. 8B illustrates another experiment also performed in a denervated preparation. The closed circles correspond to the resistance of the membrane after immersion for 20 min in a solution of eserine (5 μg/ml). No change of the resistance was observed as it was usual when small concentrations of eserine were used. Immediately afterwards, 0.2 μg/ml of acetylcholine were added to the solution. The resistance of the membrane was determined again 30 min (crosses) and 50 min (triangles) after the addition of acetylcholine. The resting potential decreased in 50 min from 86 to 80 mV and the spike from 146 to 114 mV. The resistance of the membrane did not change. Another measurement was performed 66 min after the addition of acetylcholine. The resting potential was 75 mV; the spike measured about 76 mV and was not propagated. The membrane resistance increased somewhat as shown by the squares in Fig. 8B. In summary, the resistance of the membrane at rest did not change significantly in two of the experiments performed with acetylcholine; in six others the membrane resistance increased 25% or more. Similar increases in resistance were observed in four experiments performed with carbamylcholine and three experiments with butyrylcholine (to be published). These observations are in contrast with the rapid and considerable decrease of the resistance of the membrane at rest (to 50% or less of the control value) caused by an excess of potassium (unpublished results).

DISCUSSION

It has been demonstrated that acetylcholine reduces the resting potential measured across the innervated membrane of the electroplaque and decreases progressively the

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amplitude of the action potential. It has also been shown that the innervated membrane is formed by at least two physiologically different regions: the postsynaptic areas and the rest of the membrane[7]. An elementary question arises, therefore, regarding the site of action of externally applied acetylcholine.

It was previously described that the action potential is produced by the non-synaptic region of the innervated membrane[7]; these non-synaptic areas are directly affected by acetylcholine, for this compound completely blocks the action potential. The inhibition of the spike by acetylcholine may occur without change of the resting potential (Fig. 4) and consequently it is not mediated by depolarization of the membrane.

On the other hand, the observed depolarization can take place at each of the two components of the innervated membrane. The electrical recordings are not sufficiently discriminative to differentiate between depolarization of the postsynaptic region and a consequent electrotonic spread to the neighboring areas of the local negativity or a simultaneous depolarization of the whole membrane. The electrotonic spread of activity confined to the postsynaptic region is clearly demonstrated by experiments in which the postsynaptic potential was studied. The latter was practically of the same magnitude and duration when recorded by intracellular electrodes regardless of the position of the electrode inside the cell[7]. This finding is not surprising when it is realized that the innervated membrane makes synaptic contact with nerve fibers every 4 to 30 microns[15].

Since a method based purely on the recording of the electrical activity to locate the depolarization did not appear promising, another procedure was designed. The experiments performed with compounds that block with high relative specificity the synaptic region in small concentration, offer an answer to this problem. The degree of inhibition of the postsynaptic potential was used to evaluate the effect of the drug on the post-synaptic areas. These is a close parallelism between the block of the synaptic transmission caused by compounds such as *d*-tubocurarine and their antagonistic properties with regard to the depolarizing action of acetylcholine. Furthermore, as shown in Fig. 5 and as mentioned in RESULTS, all the compounds tested so far that block selectively the synaptic region, may antagonize the depolarizing action of acetylcholine without modifying the effect of the latter on the spike. As shown above, the inhibition of the action potential takes place by direct effect of acetylcholine on the nonsynaptic membrane. It may be concluded that acetylcholine applied externally causes depolarization only at the postsynaptic membrane of the electroplaque. This local depolarization propagates electrotonically to the neighboring regions. This conclusion is in agreement with experiments carried out in striated muscle. There, acetylcholine depolarizes only the end plate region; it has no effect on the rest of the membrane when applied either from the inside of the cell or from the outside.

The findings just described are especially important with regard to the measurement of the resistance of the membrane at rest. It was described above that acetylcholine usually increases this resistance. On the other hand, KATZ[16] demonstrated in frog striated muscle that the end plate potential which, as generally accepted, is produced by acetylcholine, decreases the resistance of the postsynaptic region. A probable explanation of this apparent contradiction arises from the histological structure of the electroplaque. The area of contact between a nerve terminal and the

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postsynaptic membrane is very small in the electroplaque. The sum of the areas of all the postsynaptic regions is surely a small percentage of the total area of the whole innervated membrane[15]. It may be expected that even important changes of the resistance of the postsynaptic region will modify only slightly the overall resistance measured across the innervated membrane. Furthermore, there is ground to think that the increased resistance observed after exposure to acetylcholine is probably due to the effect of the compound on the non-synaptic membrane. Quaternary ammonium salts that do not depolarize such as tetra-ethyl, tetra-*n*-propyl and tetra-*n*-butyl-ammonium or as choline that reduces slightly the resting potential, produce a constant and important increase of the membrane resistance (unpublished observations). Probably, these compounds do not decrease the resistance of the postsynaptic region since they do not depolarize. Therefore, any increase of resistance of the non-synaptic membrane produced by these compounds should be more evident than in those cases in which the resistance at the postsynaptic regions is simultaneously decreased.

It may be accepted that the action potential is the result of a net influx of sodium as demonstrated in squid axon[12, 17]. In a similar cell, when the electrochemical potential of sodium is the same in both sides of the membrane, no net influx of sodium will take place during activity. The condition of equilibrium may be reached experimentally, by keeping the transmembrane potential at an adequate level by means of a superimposed electric field[14]. However, quite different magnitudes may be obtained according to the physiological state of the membrane as shown in Fig. 7, although the concentration of sodium outside and probably inside the cell was not changed. It is likely, therefore, that under the conditions of Fig. 7 not the electrochemical potential of sodium is measured, but only the transmembrane potential at which no net influx of sodium occurs. The influx of any ionic species may be defined as the product of the total driving force by the concentration of the ion in the extracellular fluid, and by its mobility in the membrane[18]. Since under the experimental conditions of Fig. 7 neither the force nor the concentration of sodium ions have changed, a decrease of its mobility in the membrane can explain satisfactorily the results obtained. It appears, therefore, that acetylcholine has decreased the permittivity to sodium of the non-synaptic membrane, at least during the action potential. Furthermore, the decrease of the rate of rise of the spike produced by acetylcholine, suggests that the change of the membrane that allows the increased permittivity to sodium during the first phase of the action potential is markedly slowed down by this compound.

It may be noted that an increased permittivity to Cl, as shown by SOLLNER[19] in model membranes, would produce a similar result. However, since the resistance of the membrane is not changed, this increase in permittivity seems improbable.

The question may be raised as to the implications of the findings reported in regard to the physiological role of acetylcholine in the propagation of the spike along a membrane. Acetylcholine applied externally has an effect on end plates, heart muscle fibers, smooth muscle and gland cells similar to that observed by stimulation of the innervating nerves. It depolarizes postsynaptic membranes, but, as is well known, acetylcholine has little or no pharmacological action on other membranes such as nerve or striated muscle, that propagate spikes. Under the present experimental conditions, acetylcholine apparently does not depolarize the nonsynaptic membrane of the electroplax, but causes the progressive reduction of the amplitude

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and duration of the action potential. It decreases the rate of rise of the spike and seems to reduce the sodium permeability of the active membrane without decreasing the resistance of the membrane at rest. These effects seem to be at variance with those which one would expect on the basis of the hypothesis proposed by NACHMANSOHN[20] as to the detailed mechanism of acetylcholine action; they are all in the direction of blocking and decreasing the electric response in contrast to the effects at the postsynaptic membrane. Without going into details of possible alternative interpretations, the main problem is whether acetylcholine applied externally to the conducting membrane has the same effect as acetylcholine released internally within a specific temporal and spatial arrangement. There is no direct experimental evidence available as to this difficult problem and further work is necessary before a conclusive answer may be offered.

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SUMMARY

1. The effect of acetylcholine on the electroplax of electric eel has been reinvestigated by means of a preparation which allows a more rapid diffusion of the drug to the membrane of the studied cell than previously used.

2 Acetylcholine depolarizes the innervated membrane and simultaneously reduces the amplitude and duration of the action potential which eventually disappears.

3. The efficiency of acetylcholine in normal or denervated electroplax is increased about 300 times when eserine is added (5 to 10 μg/ml).

4. The action of externally applied acetylcholine is not spontaneously reversible. However, small concentrations of *d*-tubocurarine cause a rapid recovery of the resting potential which may be accompanied by partial recovery of the action potential. The depolarization caused by acetylcholine is also reversed by procaine and some quaternary ammonium compounds such as tetraethyl tetra-*n*-propyl and tetra-*n*-butylammonium.

5. *d*-Tubocurarine antagonizes the depolarization caused by acetylcholine and inhibits the postsynaptic potential in a parallel degree. All the compounds studied that block the synaptic transmission antagonize the depolarizing action of acetylcholine at a concentration far below that necessary to produce an effect upon the action potential.

6. Acetylcholine produces a significant increase of the excitability of the directly stimulated membrane only when an important depolarization is present. It is not clear whether the decrease in threshold is due to a direct effect of acetylcholine on the non-synaptic membrane or whether it is mediated through the depolarization.

7. As previously mentioned acetylcholine decreases the amplitude of the action potential and simultaneously reduces the magnitude of the transmembrane potential sufficiently to prevent a net influx of sodium during activity. It also reduces the rate of rise of the action potential.

8. Acetylcholine does not decrease the resistance of the membrane at rest and in the majority of the experiments a significant increase of this resistance has been measured.

9. It is concluded that externally applied acetylcholine apparently depolarizes only the postsynaptic region. At the non-synaptic membrane acetylcholine depresses the action potential, possibly by reducing the permeability of the active membrane to sodium.

10. The results are discussed with regard to current theories dealing with the physiological role of acetylcholine.

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