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# THE ACTION OF ACETYLCHOLINE AND CHOLINESTERASE INHIBITORS ON SINGLE AXONS OF THE LOBSTER*

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**Abstract**—Acetylcholine (ACh), physostigmine and related substances have been tested for their effects on the electrical activity of single giant axons of the circumesophageal connectives of the lobster. Acetylcholine and physostigmine in concentrations of $5 \times 10^{-3}$ to $1 \times 10^{-2}$M depolarize the membrane. The simultaneously recorded action potential is prolonged, and with progressive depolarization reversible block of conduction occurs. Both compounds cause initially spontaneous or repetitive firing. Neostigmine, 3-hydroxyphenyltrimethylammonium and edrophonium prevent the effect of ACh without having an effect of their own on membrane or action potential. Choline, cholinethiol and phenyltrimethylammonium do not antagonize the action of ACh, nor do they change the electrical parameters of the axon. Curare depolarizes the membrane by 15 mV, but does not cause block of conduction nor does it inhibit the ACh effect. These observations are discussed and possible mechanisms of action are suggested.

ALTHOUGH it is known that acetylcholine (ACh) is present in peripheral nerve fibers,[1,2] many problems remain as to its precise mode of function. Until a few years ago, no effects of ACh on the electrical parameters of peripheral nerve fibers were obtained. This was the basis for the conclusion that it has no part in excitable membranes of the peripheral nerve in contrast to its well established role at the nerve terminal. The demonstration that acetylcholinesterase (AChE) was present in most peripheral nerve fibers in high concentrations and located at or near the membranes was not considered sufficient evidence for the function of ACh.[3–5] Furthermore, the existence of structural barriers preventing ACh from reaching the conducting membrane was ignored. More recently, however, direct effects of ACh on the electrophysiological properties of the C fibers of the rabbit vagus and of the walking leg nerve of the lobster were demonstrated.[6,7] These fibers are multifiber preparations with a mixed fiber population and offer therefore a number of pitfalls.[7,8] It thus appeared important to use a single fiber preparation.

The present experiments deal with the action of ACh and related substances recorded from giant axons of the circumesophageal connectives of the lobster (*Homarus americanus*).

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## METHODS

The technique used was essentially the same as that described by Dalton[9] and by Tobias and Bryant.[10] The circumesophageal connectives were excised from the lobster and tied with cotton threads between the cerebral and subesophageal ganglion. After removal of the sheath, the bundle was placed into a lucite chamber and the cotton thread clamped down on either end. Thus it was easy to regulate the tension and position of the bundle. At least 7 giant axons could be separated from the bundle. They varied in diameter, but each was large enough to be impaled with a microelectrode. Three of the largest axons, grouped together, and situated along the surface were used. No qualitative difference in drug response was found among the different axons. Each experiment was performed on the same axon and, if possible, with a single impalement. The resting potential was recorded continuously with a Varian ink recorder and the action potential was monitored on a Tektronix storage oscilloscope. The glass electrodes were filled with 3 M KCl and had a resistance of 7–12 MΩ. The stimulating electrodes were insulated with epoxy resins, except for the tips, and mounted on a micromanipulator. The cathode was placed on or near the axon to be impaled. The action potentials were always obtained with the threshold stimulus strength at 0·1 msec duration and were considered blocked if no spike could be elicited with a stimulus strength of 150 V at 0·1 msec duration. The nerves were bathed in artificial sea water of the following composition in mM/l: NaCl, 423; KCl, 9; $CaCl_2$, 9·3; $MgCl_2$, 23; $MgSO_4$, 25·5; $NaHCO_2$, 2·2; Tris buffer, 1. The pH was adjusted to 7·8 The temperature varied between 23° and 26°. The flow rate of the sea water through the chamber was approximately 2–3 ml/min, the chamber volume being 3 ml. Some of the differences in resting and action potentials may be due to the quality and size of the lobsters. The axons of larger lobsters had a greater diameter than those of smaller lobsters and were more consistent in their electrophysiological responses. In general, larger axons are surrounded by more connective tissue, which might reduce the danger of injury during dissection and subsequent handling. In evaluating the drug effects, only those preparations were used where the initial action potential showed an overshoot and a duration of 1 msec or less. Control experiments with choline bromide and sucrose showed that the increase in molarity due to the relatively high concentrations of compounds added had no effect on either the action or resting potential.

AChE activity was determined by the Hestrin colorimetric technique[11] with ACh at $1 \times 10^{-2}$ M concentration as a substrate. For the enzyme measurements, sea water was buffered with $1 \times 10^{-1}$ M Tris adjusted to pH 7·6. The desheathed axons were preincubated with the selected concentration of inhibitor for 30 min and then placed into the final incubation media, containing ACh and the inhibitor. Control nerves were incubated in ACh without inhibitor. Readings were taken at hourly intervals over a 6-hr period.

All compounds were obtained commercially with the exception of cholinethiol, which was kindly provided by Dr. H. Mautner of Yale University.

3-Hydroxyphenyltrimethylammonium was prepared by Dr. S. Ginsburg, for which we are very grateful. Choline bromide and ACh bromide were recrystallized before use.

## RESULTS

*Action of ACh and some related substances.* When the sea water bathing the nerve is

switched to a solution containing ACh at a concentration of $1 \times 10^{-2}$ M, there is a decrease in the resting potential (Fig. 1). In 5 min, ACh depolarized the membrane 16 mV. After recovery in sea water, a renewed application of ACh depolarized at a faster rate (13 mV in 2·5 min). On return to sea water, the membrane was repolarized and conduction was restored. Exposing the preparation to ACh for a much longer

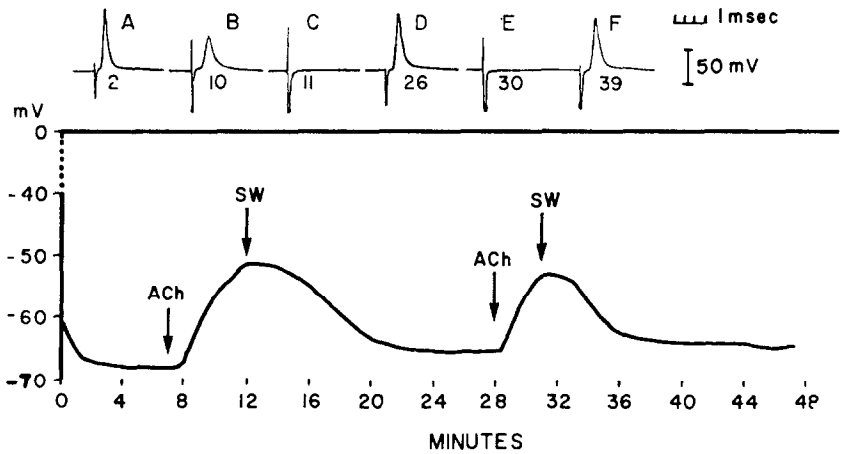


FIG. 1. Reversible effect of ACh, $1 \times 10^{-2}$M, on the membrane and action potential. ACh causes a rapid depolarization and block of conduction which are reversible in sea water. Renewed application of ACh has a faster effect. In this and the following figures the top part shows photographs of the action potentials and the bottom part, the tracing of the resting potential. The numbers below the action potentials correspond to the time scale of the abscissa. Changes of solution are indicated by arrows. SW = sea water.

time (60 min) causes almost complete depolarization, as can be seen in Fig. 2. Repeated impalements of different axons in the same bundle always showed this depolarizing action of ACh. After long exposure to ACh and subsequent return to sea water, either no recovery or only a transient, partial recovery of the resting potential was seen. Steady state potentials were never observed with any concentration of ACh; as long as the axons were exposed to ACh they continued to depolarize. At a lower concentration of ACh, the rate was slower; however, the rates of depolarization from axon to axon were too variable for an attempt to obtain concentration–activity relationships. In general, axons with low action and resting potentials depolarized at a faster rate than those with larger potentials.

When axons had a large initial action potential (110 mV or greater), a fast small depolarization of about 6–10 mV was seen within a few minutes after application of ACh. During this phase of the response to ACh, the axon seemed to be in a hyperexcitable state. In some experiments repetitive and spontaneous firing was observed only during this initial, small depolarization; in others, the spike amplitude was slightly increased. After the repetitive firing stopped, the membrane repolarized by 2–5 mV and the action potential increased in duration and started to decrease in amplitude. This repolarization was followed by a subsequent depolarization, usually at a slower rate than the initial one. The action potential was blocked, often immediately at the onset of this second depolarization. Good recovery of both the action and membrane

potentials was observed when the bathing solution was changed to sea water right after the block of conduction. Acetylcholine caused repetitive firing in 10 of 32 experiments and spontaneous firing was seen in 2 of them. Repetitive firing was seen twice on the same day while the preparation was in normal sea water. This repetitive firing lasted much longer than the one observed in the presence of ACh and did not depend on a depolarization. It was never seen on preparations where it occurred after addition of ACh. It should also be emphasized that spontaneous activity in the presence of ACh occurred only in axons with action potentials of high amplitude and short duration.

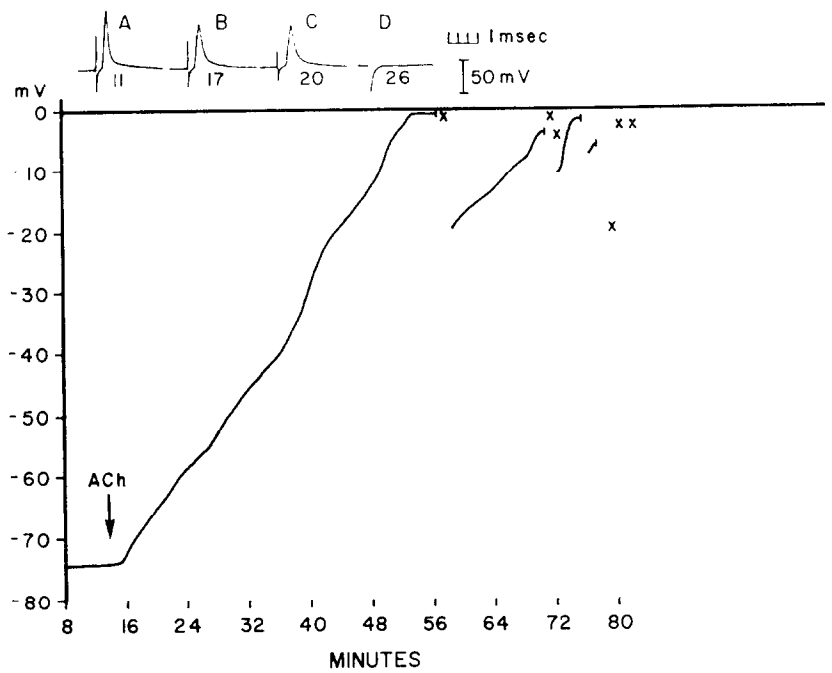


FIG. 2. Irreversible effects of prolonged application of ACh, $1 \times 10^{-2}$M. Prolonged application of ACh causes an irreversible depolarization and block of conduction. The crosses mark different impalements of the same or different axons in the bundle.

Choline at concentrations as high as $5 \times 10^{-2}$ M has no effect on either the action or resting potential. Cholinethiol, which in contrast to choline depolarizes synaptic junctions,[12] has no effect on these axons. The normal depolarizing response to ACh is observed in the presence of choline as well as cholinethiol. Trimethylbutylammonium at a concentration of $1 \times 10^{-2}$ M applied over a period of 60 min had no effect on action and resting potentials.

*Effects of curare and tetracaine.* Curare, $1 \times 10^{-2}$ M (*d*-tubocurarine chloride), causes a reduction of the resting potential. The mean depolarization found in 5 experiments was 14 mV (13–17 mV). In contrast to the depolarization observed with ACh, the potential reached a steady state (Fig. 3). The action potential was only slightly reduced, and sometimes a transient increase in spike height during the initial depolarization was seen. In two experiments conduction was blocked, but only after

the preparations were returned to normal sea water subsequent to the exposure. In one of these experiments conduction was restored after prolonged washing. Curare does not antagonize the action of ACh, as can be seen in Fig. 3. After application of curare, the membrane depolarizes and reaches a steady state. Addition of ACh causes a further depolarization, an increase in duration and finally block of the action potential.

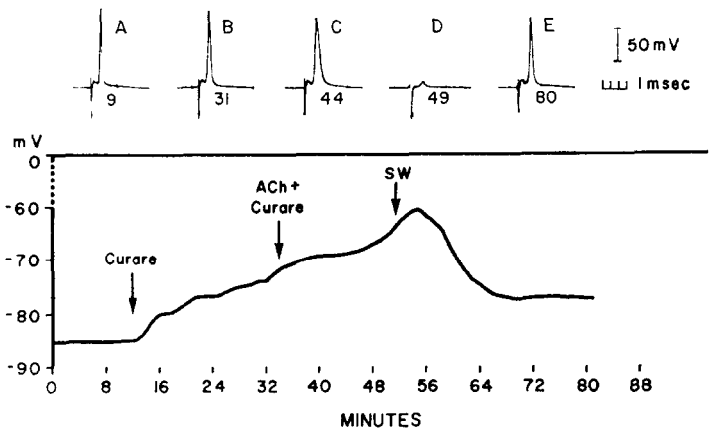


FIG. 3. Effect of curare on membrane and action potential. After 20 min in curare, $1 \times 10^{-2}$M, the membrane is depolarized 11 mV, while the action potential remains unchanged (B). Block by equimolar concentration of ACh is not prevented by curare (D). The curare and ACh effects are reversible (E).

Tetracaine at $5 \times 10^{-3}$ M depolarizes the membrane similar to ACh. At concentrations where it had no effects of its own, it did not inhibit the action of ACh.

*Effects of physostigmine on the resting and action potential.* Fig. 4 demonstrates the effect of $1 \times 10^{-2}$ M physostigmine. It caused a depolarization of the membrane by

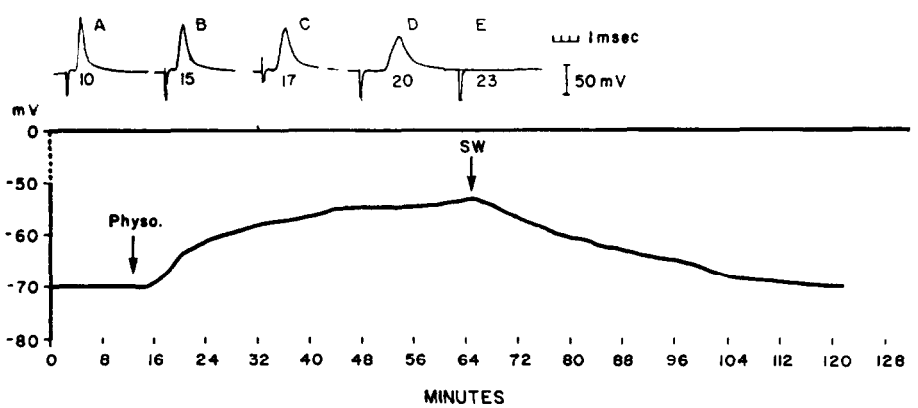


FIG. 4. Effect of prolonged application of physostigmine, $1 \times 10^{-2}$M, on the action and membrane potential. As the membrane potential decreases, the descending phase of the action potential is prolonged (B, C, D). After 13 min, block of conduction occurs (E). On return to sea water the membrane repolarizes, while the action potential does not return.

16 mV over a period of 25 min, after which it reached a steady state. This steady state remained unchanged for 30 min. Rinsing with sea water restored the initial resting potential. The action potential was blocked after 10 min and did not recover in sea water. The duration of the action potential, before it is blocked, is increased 3- to 4-fold. This prolongation is more pronounced than that caused by ACh. If the axons are returned to sea water before block of conduction, full recovery of the action potential is usually observed. The rate of recovery of the resting potential is slower than after ACh. The threshold concentration for physostigmine was $2{\cdot}5 \times 10^{-3}$ M. In 5 of 12 experiments physostigmine caused repetitive firing. Fig. 5 demonstrates the

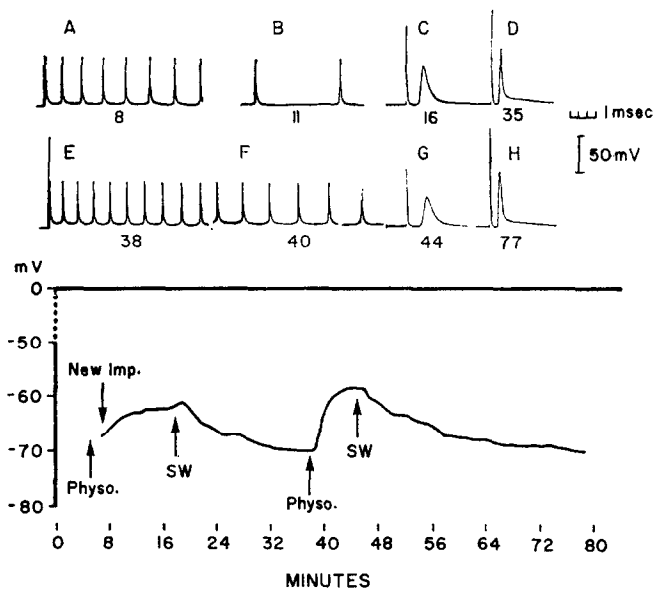


FIG. 5. Repetitive and spontaneous firing caused by physostigmine, $1 \times 10^{-2}$M. The inital impalement was lost and the same axon was reimpaled. With the onset of depolarization, repetitive firing (A and E) and spontaneous firing (B and F) can be seen. Note that on reapplication of physostigmine the rate of depolarization is faster than that of the initial one. The effects on membrane and action potential are reversible. Time calibration: 1 msec = C, D, G, H; 5 msec = A, B, E, F.

effects of repeated applications of physostigmine ($1 \times 10^{-2}$ M). Repetitive (A and E) and spontaneous (B and F) activity were observed at the onset of the depolarization; this activity lasted for about 5 min. In other experiments physostigmine, $1 \times 10^{-2}$ M, depolarized the membrane until it reached a steady state potential. When ACh, $1 \times 10^{-2}$ M, was added to the same concentration of physostigmine, no further depolarization was observed. The response to physostigmine seems to be more uniform than that of ACh; this might be due to the smaller depolarization, which never exceeded 15–16 mV regardless of how long physostigmine was applied. Lower concentrations of physostigmine, which had no effect on the electrical parameters, did not potentiate the effect of ACh.

*Modification of the action of ACh by neostigmine and its analogs.* Neostigmine at concentrations as high as $2 \times 10^{-2}$ M had no effect on the action or resting potential in 40 min, nor did it potentiate the action of ACh, but instead prevented this effect

as seen in Fig. 6. Addition of neostigmine plus ACh to a nerve preparation that had been depolarized and blocked by ACh resulted in a repolarization of the membrane and restoration of conduction. The removal of neostigmine from the test solution allowed ACh to act again. In similar experiments the recovery of the resting and action potential is not as complete as that seen in Fig. 6. Addition of ACh to preparations which had been preincubated in $1 \times 10^{-2}$ M neostigmine for 30 min caused no change in resting or action potential. This action of neostigmine is reversible.

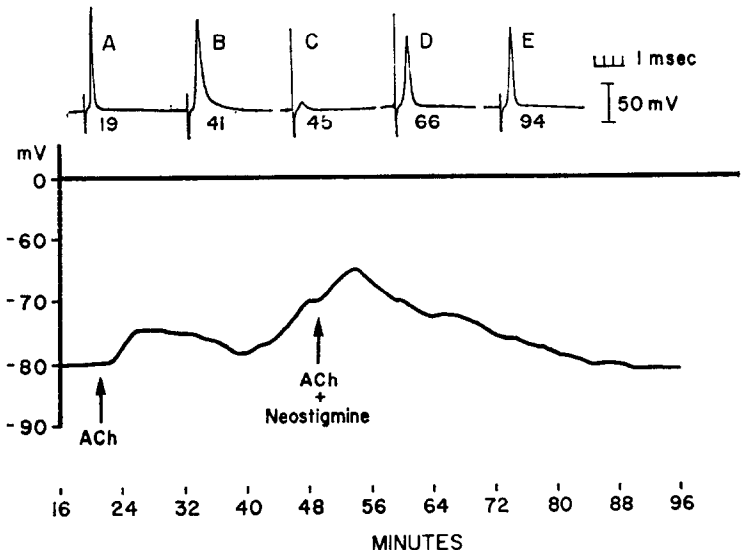


FIG. 6. Antagonism by neostigmine of the action of ACh on the membrane and action potential. ACh, $1 \times 10^{-2}$M, depolarizes the membrane and causes block of conduction (C). Addition of an equimolar concentration of neostigmine to the ACh at a time when block of conduction has occurred not only repolarizes the membrane but also restores conduction (D, E). This concentration of neostigmine has no effect by itself on either the membrane or action potential.

When the hydrolysis product of neostigmine, 3-hydroxyphenyltrimethylammonium, was tested in experiments similar to those described for neostigmine, the same effects were observed. If the bundle is preincubated with $1 \times 10^{-2}$ M 3-hydroxyphenyltrimethylammonium for 30 min, there is no change in resting or action potential when ACh is added in the same concentration.

Phenyltrimethylammonium in even higher concentrations than ACh had no effect by itself, nor did it prevent the action of ACh, as seen in Fig. 7. Acetylcholine even caused repetitive and spontaneous firing in its presence.

Edrophonium, another analog of neostigmine, where one methyl of the quaternary nitrogen is replaced by ethyl, also has an inhibitory effect, as seen in Fig. 8. After ACh depolarized the membrane and blocked the action potential, the addition of an equimolar concentration of edrophonium caused a repolarization of the membrane to its initial potential. Simultaneously, the action potential recovered to its original amplitude. After the removal of edrophonium from the bath, ACh which had been

present throughout the experiment again caused block of conduction and depolarization of the membrane, which were reversed in sea water.

The effects of these cholinesterase inhibitors on hydrolysis of ACh by intact axons are shown in Table 1. The intact desheathed nerve had an initial activity of 810 $\mu$mole ACh hydrolyzed/g nerve/hr. It was possible to inhibit completely the hydrolysis of

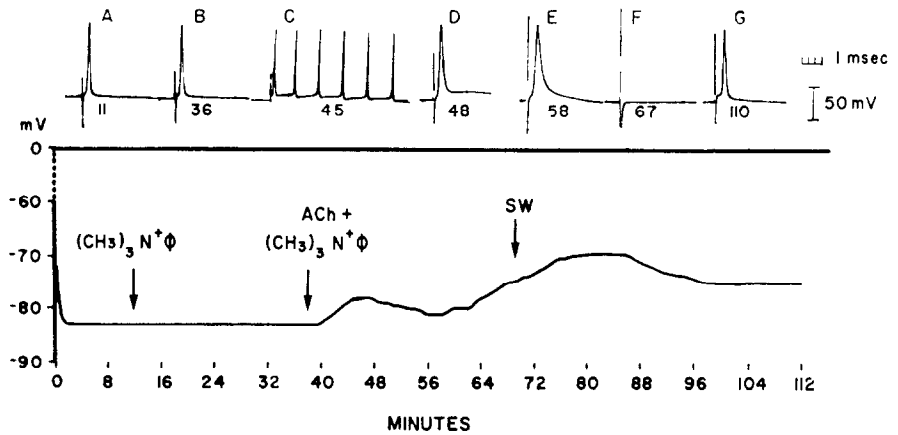


FIG. 7. Action of ACh in the presence of phenyltrimethylammonium. In spite of the presence of $1 \times 10^{-2}$M phenyltrimethylammonium, which has no effect of its own, ACh in a $1 \times 10^{-2}$M concentration causes repetitive firing during the initial phase of the depolarization (C) and block of conduction (F), which is reversed in sea water (G). Time calibration: C = 5 msec; all others = 1 msec.

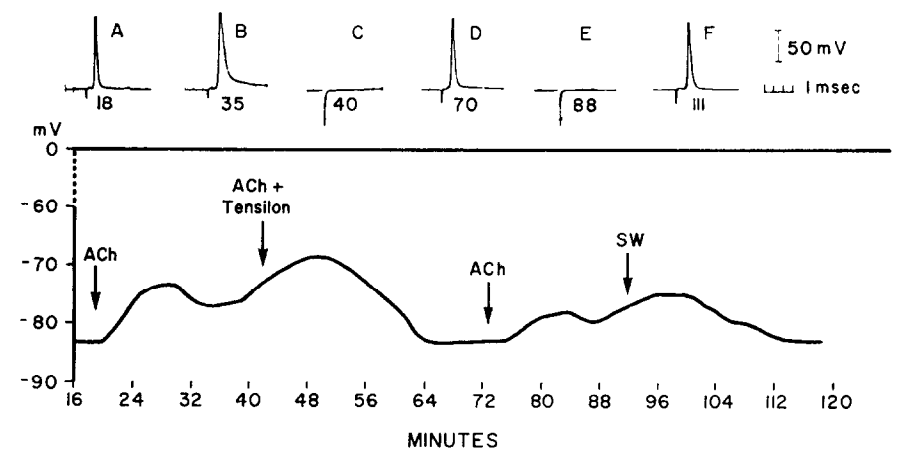


FIG. 8. Complete reversal of ACh action by tensilon (edrophonium). ACh, $1 \times 10^{-2}$M. depolarizes the membrane and blocks conduction. Addition of $1 \times 10^{-2}$M tensilon repolarizes the membrane and restores conduction in the presence of ACh. After removal of tensilon, ACh depolarizes the membrane and blocks conduction (E). These effects are reversible in sea water (F).

ACh in intact nerve when inhibitor concentrations between $1 \times 10^{-3}$ and $1 \times 10^{-2}$ M were used; the only exception was trimethylphenylammonium, which would require a concentration higher than $1 \times 10^{-2}$ M to inhibit the enzyme.

## DISCUSSION

This paper reports for the first time a direct action of ACh on the electrical parameters of a single axon, without any preceding chemical treatment. ACh reversibly depolarizes the axonal membrane and thus blocks conduction. At the onset of the ACh depolarization, repetitive and spontaneous firing was observed in a number of experiments. These actions are of interest since Nachmansohn has postulated that

TABLE 1. COMPARISON OF INHIBITORY STRENGTH OF SOME ChE INHIBITORS ON THE ENZYME ACTIVITY OF INTACT LOBSTER NERVES*

| Inhibitor | No. of experiments | Concn (M) | Hydrolysis of ACh % remaining activity |
|---|---|---|---|
| Physostigmine | 6 | $1 \times 10^{-6}$ | 51·0 (39–72) |
| | 5 | $1 \times 10^{-5}$ | 25·0 (17–34) |
| | 6 | $1 \times 10^{-4}$ | 8·0 (0–17) |
| | 6 | $1 \times 10^{-3}$ | 5·0 (0–10) |
| | 6 | $1 \times 10^{-2}$ | 0·0 (0) |
| Neostigmine | 5 | $1 \times 10^{-5}$ | 52·0 (42–64) |
| | 4 | $1 \times 10^{-4}$ | 37·0 (35–42) |
| | 6 | $1 \times 10^{-3}$ | 0·0 (0) |
| Edrophonium | 5 | $1 \times 10^{-5}$ | 47·0 (37–53) |
| | 6 | $1 \times 10^{-4}$ | 35·0 (26–43) |
| | 8 | $1 \times 10^{-3}$ | 11·0 (0–16) |
| | 5 | $1 \times 10^{-2}$ | 0·0 (0) |
| 3 Hydroxyphenyltrimethyl-ammonium | 4 | $1 \times 10^{-4}$ | 33·0 (26–39) |
| | 5 | $1 \times 10^{-3}$ | 12·0 (8–17) |
| | 5 | $1 \times 10^{-2}$ | 0·0 (0) |
| Trimethylphenylammonium | 6 | $1 \times 10^{-3}$ | 66·0 (61–76) |
| | 5 | $1 \times 10^{-2}$ | 27·0 (17–40) |

* The AChE activity of intact control nerves was 810 ± 25 μmole ACh hydrolyzed/g nerve/h. Substrate concentration was $1 \times 10^{-2}$M ACh; numbers in parentheses indicate range of values.

ACh triggers the permeability changes that occur during the conduction of an impulse.[1] The action of curare is similar to that reported on the lobster walking leg nerve[7] and rabbit vagus,[13] except that it did not antagonize the action of ACh. Rather than antagonize ACh, as seen on synaptic junctions, curare had a small depolarizing action by itself. Even after long exposure, it does not block conduction. A block of electrical activity was observed in only 2 of 5 experiments after removal of curare. In venom-treated squid axons, curare potentiates the effect of ACh and increases simultaneously the penetration of ACh into the axoplasm.[14] The curare effects on the lobster axons are only seen with high concentrations. The fact that curare reacts with quite a few macromolecules, like mucopolysaccharides, and with a variety of proteins may account for its action. An explanation for these observations requires further studies.

Depolarization and spontaneous activity caused by physostigmine on single axons have not been described before, although block of conduction has been seen on a variety of nerve preparations, myelinated and unmyelinated.[15] The membrane, after being depolarized by 10–15 mV, reached a steady state potential. The depolarizations caused by ACh were larger than those caused by physostigmine and never reached a

steady state unless complete depolarization occurred. No potentiation of ACh was found with neostigmine, 3-hydroxyphenyltrimethylammonium, physostigmine or edrophonium. These three inhibitors do not affect the resting or action potential, in contrast to physostigmine; however, they always prevent the action of ACh. This latter effect is seen regardless of whether they are applied before adding ACh or after ACh has acted. Even in the presence of ACh these compounds repolarize the membrane and restore conduction. Once they are removed from the test solution, ACh becomes effective again. These observations agree with those of Armett and Ritchie on non-myelinated C-fibers of the rabbit vagus.[6]

It is apparent that there are striking qualitative differences in the pharmacological action of the compounds when tested on the synaptic junction or on the lobster giant axon. If one postulates a common physiological role of ACh at junctions and at conductive membranes, these pharmacological differences have to be explained. The difference in structure of the membranes and the structures surrounding the axon proper could modify the pharmacological responses. More and more evidence of the complexity and diversity of membranes is accumulating, e.g. charge distribution and functional substructures. However, at this time we have no definite information on the fine structure and molecular arrangement of the membranes we are dealing with, so that this factor is a complete unknown for the interpretation of our data. In venom-treated squid giant axons, ACh and curare blocked conduction, which was not observed otherwise. Choline, neostigmine and physostigmine prevented the action of ACh, which was interpreted as being due to a competition for penetration sites, which also would account for the failure of AChE inhibitors to potentiate the effect of ACh.[16] This interpretation might also explain the effects as seen on the lobster axons; however, choline does not prevent ACh action as it does on the squid axon.

Physostigmine is the only reversible AChE inhibitor which depolarizes the membrane of the lobster axon and blocks conduction. As was shown 20 yr ago with the squid axon,[1] physostigmine, being a tertiary nitrogen compound, can penetrate into the axon and reach the enzyme, while neostigmine does not. A possible explanation for the physostigmine action on the lobster axon when used in high concentrations may be that it blocks conduction by inhibition of AChE, which in turn leads to an accumulation of ACh causing the depolarization and repetitive firing. Before the block is observed, the action potential decreases in amplitude and increases in duration. These nerves contain 9 $\mu$g ACh/g nerve and are able to synthesize 180 $\mu$g ACh/g nerve/hr.[17] Whether this concentration is large enough to explain the physostigmine effect through an accumulation of ACh cannot be decided as yet, since we do not know the critical concentrations of ACh in the region of the receptor. These observations—the failure of physostigmine to depolarize to the extent seen with ACh and the inhibition of the effect of externally applied ACh by physostigmine—could be due to competition between physostigmine and ACh for receptor sites which might be different from the enzyme. Therefore no potentiation of ACh can be observed. Evidence has been accumulated that at least on synaptic junctions the active sites of the enzyme and the receptor might not be identical.[18–20] This still leaves unanswered the question of why neostigmine, edrophonium and 3-hydroxyphenyltrimethylammonium interfere with ACh action without having an effect of their own.

It is possible that substructure and organization affect the reactions and that due to these factors the phenomena observed differ in the different preparations. It cannot

even be excluded that there are differences between different membranes on the molecular level. However, such an assumption would require more detailed chemical investigations.

Recently, Podleski and Changeux[21] have observed a depolarizing effect of ACh on the conducting membrane of an electroplax preparation which had been modified by various treatments. Their observation that di-isopropyl phosphorofluoridate prevents this response to ACh seems to be similar to our findings described above. This depolarization appeared to be due to the lowering of the intramembraneous pH. The same interpretation could apply to our observations on the lobster axon. It is possible that the action of ACh is due not to its effect on the receptor but to an accumulation of hydrogen ions within the membrane. The concentration of ACh used is very high, $1 \times 10^{-2}$ M, which is within the optimum range of substrate concentration of cholinesterase (ChE) as measured on the intact nerve. The ChE activity of the lobster axons is very high; it hydrolyzes ACh at a rate of 900–1000 $\mu$mole/g/hr as compared to 1–3 $\mu$mole/g/hr in the squid giant axon. A sufficiently high hydrogen ion concentration may rapidly accumulate and initiate reactions leading to a depolarization. When ChE inhibitors such as prostigmine, 3-hydroxyphenyltrimethylammonium and edrophonium are present, ACh hydrolysis is prevented and hydrogen ions are not formed in sufficient concentration or with sufficient speed to produce the effect. This would explain the observation that these inhibitors are completely inactive on electrical activity. Additional support for this interpretation is the fact that phenyltrimethylammonium, which is 120 times weaker as an inhibitor of AChE in solution than 3-hydroxyphenyltrimethylammonium, does not antagonize the effect of ACh on the electrical parameters of the lobster axon. However, one has to be careful in comparing results and drawing conclusions from one preparation to another; the corresponding experiments have to be done on the lobster axons and are underway.

Our findings do not yet allow us to draw any conclusions as to the mechanism of the ACh action. We do not know, for instance, whether ACh causes a specific increase in Na and K permeability during the initial stage of depolarization or a general increase in the permeability to a number of ions or whether we are dealing merely with a nonspecific pH effect. However, this preparation may permit us to test this and other problems which have arisen during the course of these investigations.

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