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REACTIONS OF ACETYLCHOLINE RECEPTOR AND ESTERASE STUDIED ON THE ELECTROPLAX

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Abstract—The effects of various compounds acting on the acetylcholine-receptor or on the acetylcholine-esterase, or on both in the synaptic membrane of the monocellular electroplax preparation have been analyzed. Additional evidence has been obtained that the active sites of these two components are different.

Physostigmine, 5×10^{-5} M, potentiates the action of acetylcholine (ACh) to its maximal effect. At higher concentrations of physostigmine the action of ACh is antagonized, thus suggesting that the two compounds compete for the active site of the receptor. In the presence of physostigmine, 5×10^{-5} M, strong spontaneous depolarizations of the membrane are triggered by indirect stimulation of 10 pulses/sec, due apparently to intramembraneous accumulation of ACh.

The depolarizing effects of acetyl-, propionyl-, and butyrylcholine are about equally strong, although butyrylcholine is a much poorer substrate of ACh-esterase than the two other esters. Dimethylcarbamate is a more potent receptor activator than monomethylcarbamate. This is in contrast to their strengths as enzyme inhibitors. The depolarizing action of neostigmine has been compared with that of some of its analogs. The potency of these compounds as receptor activators changes in a direction opposite to their strength as inhibitors of ACh-esterase in solution.

The D- and L-isomers of acetyl-β-methylcholine do not depolarize the membrane of the electroplax, but act as weak receptor inhibitors. The L-isomer is about 5 times as potent as the D-isomer. The lowest concentration of the D-isomer at which an inhibition of the response to carbamylcholine is observed is $7{\cdot}5 \times 10^{-4}$ M.

THE EFFECTS of some compounds which act on the receptor and on acetylcholinesterase (AChE) have been investigated on the single isolated electroplax. This is an extension of previous studies of structure–activity relationship.[1–3]

METHODS

The experiments were carried out on the monocellular electroplax preparation of *Electrophorus* as described before.[4, 5] The test solutions were applied only to the innervated membrane of the cell. Resting potentials were recorded with a paper-recorder; action potentials were monitored with a cathode ray oscilloscope. Single impalements, using 3 M KCl filled microelectrodes of 7–12 M Ω resistance, were maintained as long as the resting potential gave a smooth, continuous curve, which often lasted over 1 hr. The composition of the Ringer's solution was in μmole/ml: NaCl, 160; KCl, 5; $CaCl_2$ 2; $MgCl_2$, 2; NaH_2PO_4, 0·3; Na_2HPO_4, 1·2; glucose, 10. The pH was 7·0 and the temperature was 23–25°.

The "standard error" as used in this paper refers to the standard deviation divided

by the square root of the number of experiments. The S.D. was calculated from the equation:

$$\sigma = \sqrt{\left(\frac{\Sigma(y-\mu)^2}{N}\right)}$$

where σ is the S.D, μ the mean, y the observation, and N the number of experiments.

I am very grateful to Dr. Sara Ginsburg who kindly provided isovalylcholine, hexahydrobenzoylcholine, methylcarbamylcholine, dimethylcarbamylcholine, pyridostigmine, norprostigmine, 3-hydroxyphenyltrimethylammonium and the D- and L-isomers of acetyl-β-methylcholine. All other compounds used were obtained commercially.

RESULTS

Action of physostigmine. The effects of ACh and of those analogs that are hydrolyzed by AChE were tested in the presence of physostigmine, 5×10^{-5} M. In order to ascertain that this concentration gives the greatest response to ACh under the conditions used with the preparation, the steady state potentials of different concentrations of ACh were plotted as a function of physostigmine concentration (Fig. 1).

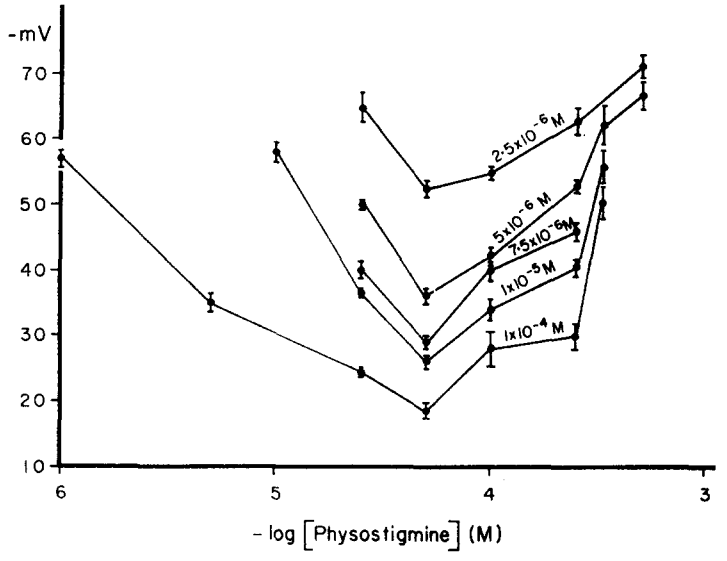


FIG. 1. Maximum potentiating concentration of physostigmine on the electroplax. Steady state potentials of different concentrations of ACh recorded on the monocellular electroplax preparation as a function of the negative logarithm of physostigmine concentration. Each curve represents a single concentration of ACh as indicated. Each value is the mean of at least 4 experiments; the vertical bars indicate the S.E. In the experiments presented in this and the following figures, the pH was 7·0 and the temperature was 23°–25°.

Each curve represents a single concentration of ACh as indicated. It is apparent from the figure that 5×10^{-5} M physostigmine is the concentration that potentiates ACh to its maximum effect. If the concentration of physostigmine is lower than 5×10^{-5} M, the potency of ACh decreases approximately proportionately to the

decrease in physostigmine concentration. As shown in Fig. 2, when different concentrations of ACh are tested in the presence of physostigmine, 5×10^{-6} M, an initial fast depolarization is followed by a slow partial repolarization to a steady state potential. The concentrations of ACh required for obtaining the same potency are approximately 10 times higher than in the presence of physostigmine, 5×10^{-5} M.

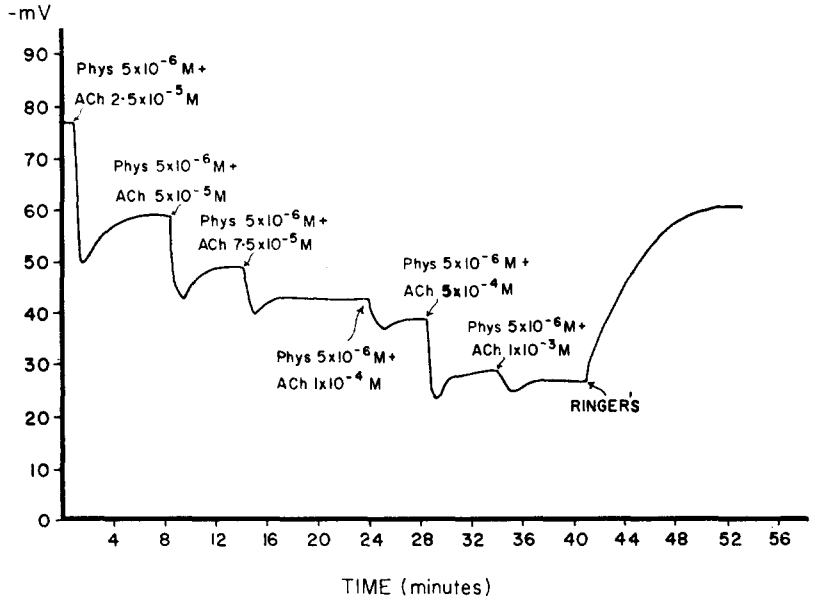


FIG. 2. The resting potential of the electroplax exposed to increasing concentrations of ACh in the presence of 5×10^{-6}M physostigmine.

The effect of increased concentrations of physostigmine on the ACh depolarizations is demonstrated in Fig. 3. The steady state potentials are plotted against ACh concentrations in the presence of different concentrations of physostigmine as indicated. Each curve is obtained by varying the ACh concentrations, keeping the physostigmine concentration constant. It can be seen that physostigmine at concentrations higher than 5×10^{-5} M acts as an inhibitor of ACh; it apparently competes for the receptor sites on which ACh acts.

Fig. 4 demonstrates an indirect action of physostigmine, 5×10^{-5} M, on the synaptic junction. The cell was stimulated through the nerve endings with anodal stimulation[6] in normal Ringer's solution at 1 and 10 square pulses/sec at threshold stimulus strength. Since the response of the Varian recorder is too slow to show an action potential, there is a sustained depolarization whose magnitude depends on the frequency of stimulation. After physostigmine, 5×10^{-5} M, was applied for 15 min to the innervated membrane, the cell was stimulated again. It can be seen that the stimulation of 10 pulses/sec causes a sudden drop in potential and a very fast spontaneous recovery which is sped up by changing the bathing solution. After the membrane potential has recovered, there is another depolarization which occurs without stimulation. After recovery, a third spontaneous depolarization and recovery occurred. This response to physostigmine is not seen consistently on all preparations. It seems

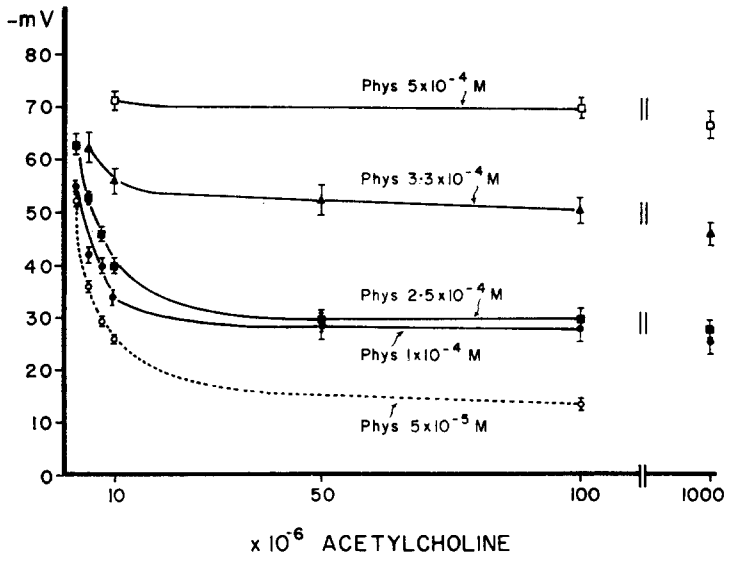


FIG. 3. Steady state potentials of the electroplax as a function of increasing ACh concentration at constant physostigmine concentration. Each value is the mean of 4 or more experiments. Vertical bars indicate the S.E. The broken line shows the effect of ACh in the presence of the optimal concentration of physostigmine.

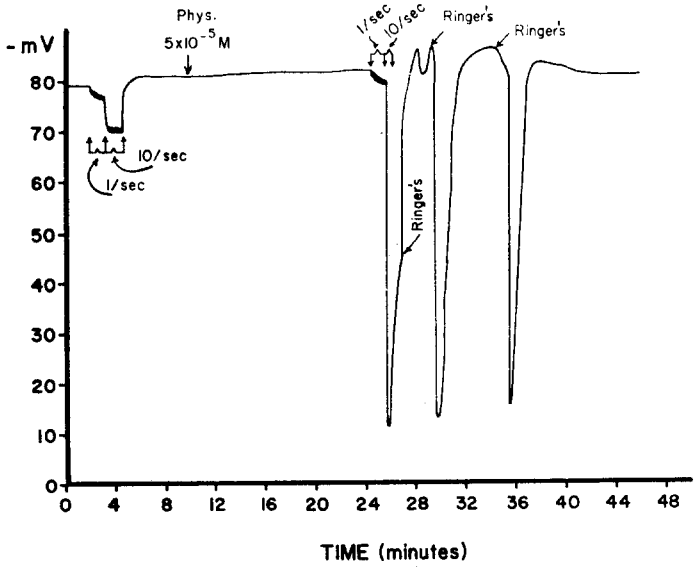


FIG. 4. Depolarization and repolarization of the electroplax in the presence of physostigmine, 5×10^{-5}M. The first depolarizations produced by 1 or 10 pulses/sec in the absence of physostigmine are small. After exposure to physostigmine for about 15 min a strong depolarization is triggered by indirect stimulation of 10 pulses/sec at threshold stimulus strength. The two following depolarizations occur spontaneously.

to occur mostly on cells that have just been dissected and have a large action potential. However, there is always an increased depolarization on indirect stimulation in the presence of physostigmine as compared to the control response in Ringer's solution. As can be seen in Fig. 5, the control responses (registered on the Varian recorder) to direct (cathodal) and indirect (anodal)[6] stimulation are identical. After the application of physostigmine, 5×10^{-5} M, the response to direct stimulation remains unchanged, but the depolarization seen with indirect stimulation is increased by a factor of 3. Before the membrane potential returns to its initial value, there is a transient hyperpolarization which does not occur after the control response in Ringer's solution or with direct stimulation in the presence of physostigmine. This has been seen consistently on the recovery curve after the cell has been exposed to carbamylcholine, ACh or other depolarizing compounds. Curare, 5×10^{-7} M, does not block the postsynaptic potential and indirectly evoked spike.[7] When this concentration of curare is added to the physostigmine solution, the increased depolarization is abolished and the response is identical with that of the control in Ringer's solution. If physostigmine again is applied without curare, the response to indirect stimulation is increased. This action of physostigmine is reversible; after 18 min in Ringer's solution the response is again identical with that of the control. After reapplication of physostigmine, the response is increased as before.

ACh analogs. There is an obvious similarity in the chemical structures of ACh and local anesthetics as Nachmansohn first pointed out.[3] The different action on the receptor seems to be due mostly to the ester group. Table 1 shows the structure and

TABLE 1. ACETYLCHOLINE ANALOGS

Compound	Structure	Rel. potency*
ACh†	$(CH_3)_3{}^+NCH_2CH_2—O—\overset{\overset{O}{\|}}{C}CH_3$	1
Propionylcholine†	$(CH_3)_3{}^+NCH_2CH_2—O—\overset{\overset{O}{\|}}{C}C_2H_5$	1·2
Butyrylcholine†	$(CH_3)_3{}^+NCH_2CH_2—O—\overset{\overset{O}{\|}}{C}C_3H_7$	1·3
Butyrylcholine		41
Isovalylcholine	$(CH_3)_3{}^+NCH_2CH_2—O—\overset{\overset{O}{\|}}{C}—CH_2CH\begin{matrix}\diagup CH_3\\ \diagdown CH_3\end{matrix}$	24
Hexahydrobenzoylcholine	$(CH_3)_3{}^+NCH_2CH_2—O—\overset{\overset{O}{\|}}{C}$—⬡	200

* In this and the following tables, "relative potency" indicates the relative concentrations required to produce equivalent effects, i.e. the production of the same steady state potentials.

† Physostigmine, 5×10^{-5} M, added.

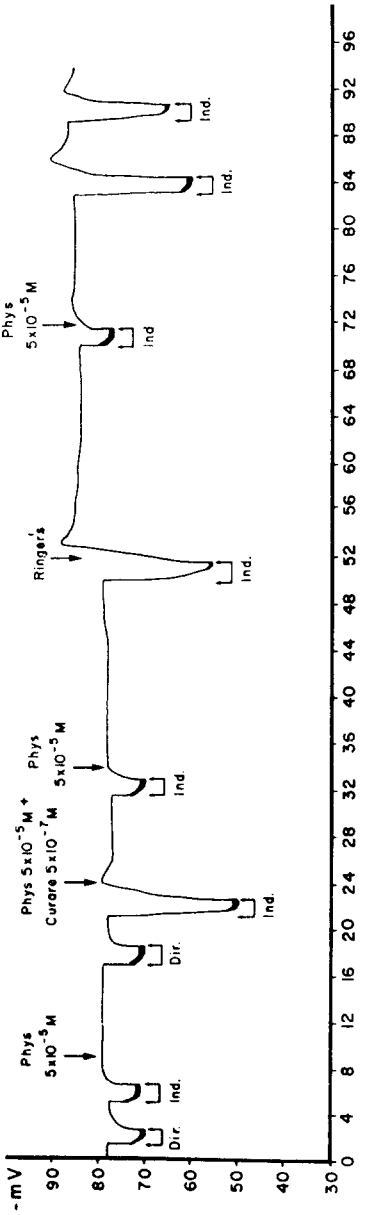


FIG. 5. Increased effect of indirect stimulation on the membrane potential of the electroplax in the presence of physostigmine, 5×10^{-5}M. Curare, 5×10^{-7}M, antagonizes this effect. Stimulation, 10 pulses/sec at threshold stimulus strength. For explanation see text.

potency of a group of compounds in which the ester group has been changed. As the basis of comparison that concentration has been used which gives a steady state potential of − 45 mV. Propionylcholine and butyrylcholine in the presence of physostigmine, 5×10^{-5} M, are only slightly weaker than ACh, by a factor of less than 2. Butyrylcholine without physostigmine still has an effect, but is 41 times weaker, indicating that it is slowly hydrolyzed by AChE. Isovalylcholine is 24 times weaker than ACh and does not require physostigmine for maximal effect. The tertiary analog of isovalylcholine does not depolarize; it blocks the postsynaptic potential at 1×10^{-2} M. Hexahydrobenzoylcholine depolarizes, but is more than 200 times weaker than ACh.[2] In Fig. 6 the steady state potentials of these compounds are plotted against

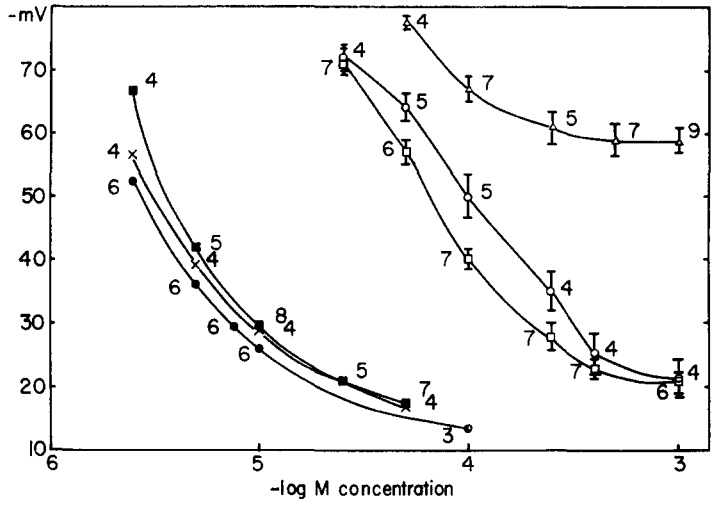


FIG. 6. Steady state potentials as a function of the negative logarithm of the concentration of some ACh analogs. The numbers next to each point refer to the number of experiments; the vertical bars indicate the S.E. In the presence of physostigmine, 5×10^{-5}M: ● = ACh; × = propionylcholine; ■ = butyrylcholine. Without physostigmine: □ = isovalycholine; ○ = butyrylcholine; △ = hexahydrobenzoylcholine.

the negative logarithms of their concentration. The curves of propionylcholine and butyrylcholine in the presence of physostigmine are very close to that of ACh. The S.E. of the measurements for these three curves is less than ± 2 mV and has been omitted. The effects of butyrylcholine and isovalylcholine without physostigmine are essentially parallel to those of ACh. As described elsewhere hexahydrobenzoylcholine depolarizes only to −60 mV; moreover, the membrane repolarizes when the concentration is further increased.

Carbamylated analogs. When the carbamyl group of carbamylcholine is methylated, the potency of the compound increases as seen in Table 2 and Fig. 7. All three compounds give a maximum depolarization to the same steady state potential of −10 to −15 mV.

Another group of compounds tested are analogs of neostigmine. Fig. 8 shows the paper-recorder tracing of an experiment testing the effect of various concentrations of neostigmine on the resting potential. With each concentration a steady state is

reached at a much slower rate than with ACh[3] or carbamylcholine.[5] The maximum depolarization reached is about — 25 mV; an increase in concentration repolarizes the membrane (see Fig. 9). In some experiments the cell was incubated in physostigmine, 5×10^{-5} M, for 10 min and then neostigmine was added at increasing concentrations. No difference could be observed in either the steady state potentials or in the

TABLE 2. CARBAMYLCHOLINE ANALOGS

Compound	Structure	Rel. Potency
Carbamylcholine	$(CH_3)_3{}^+NCH_2CH_2—O—\overset{\overset{O}{\|}}{C}NH_2$	1
Methylcarbamylcholine	$(CH_3)_3{}^+NCH_2CH_2—O—\overset{\overset{O}{\|}}{C}NHCH_3$	0·6
Dimethylcarbamylcholine	$(CH_3)_3{}^+NCH_2CH_2—O—\overset{\overset{O}{\|}}{C}—N\begin{matrix}CH_3\\CH_3\end{matrix}$	0·3

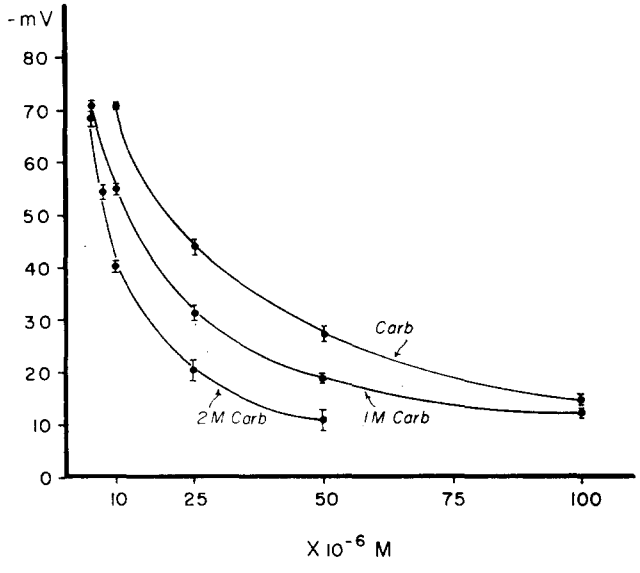


FIG. 7. Steady state potentials of carbamylcholine and two methylated analogs as a function of their concentrations. Each value represents the mean of at least 3 experiments; the vertical bars indicate the S.E.

rate of depolarization. Neostigmine, 1×10^{-5} M, which has no measurable effect of its own, potentiates ACh, but less than physostigmine, 5×10^{-5} M, and more than physostigmine, 1×10^{-5} M. Table 3 lists the relative potencies of some neostigmine analogs. Norprostigmine is slightly less potent than neostigmine, which parallels

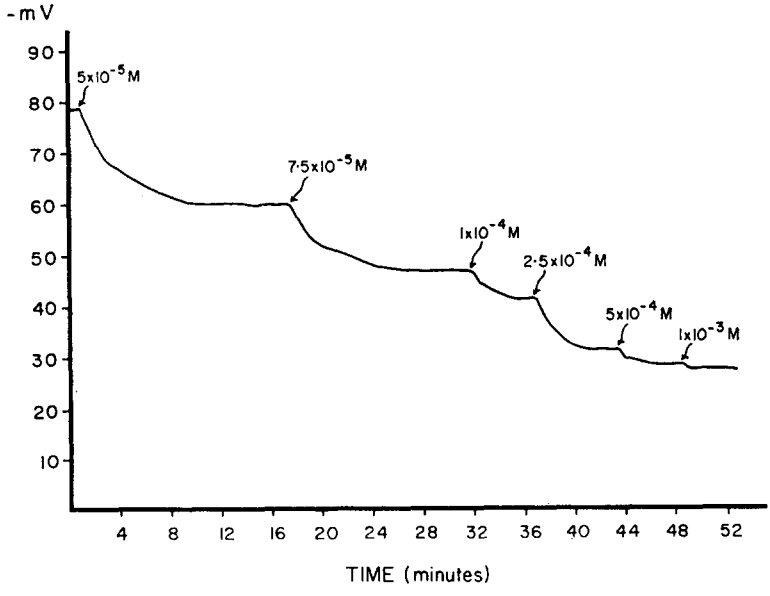


FIG. 8. Effect of increasing concentrations of neostigmine on resting potential and steady states. A representative experiment showing the changes of the resting potential; depolarizations and steady states are concentration-dependent. The arrows indicate the change of solutions.

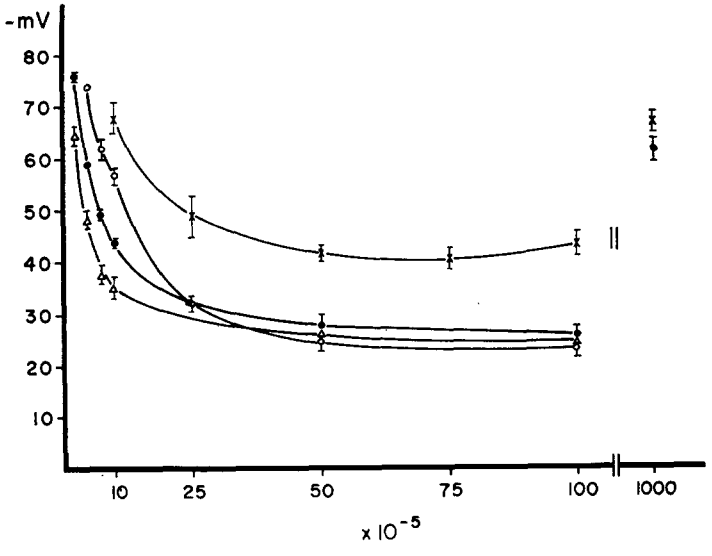
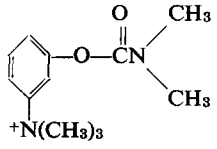
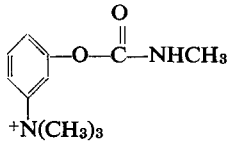
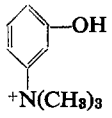
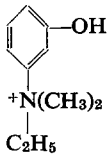
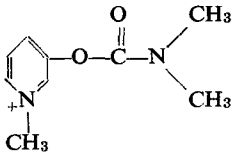


FIG. 9. Steady state potentials of neostigmine and some analogs as a function of their concentrations. Each value represents 3 or more experiments; the vertical bars indicate the S.E. ● = neostigmine; △ = 3-hydroxyphenyltrimethylammonium; ○ = norprostigmine; × = Tensilon.

the finding of the carbamylcholine series (see Table 2). 3-Hydroxyphenyltrimethylammonium, which is the hydrolysis product of neostigmine, is about twice as potent. In Tensilon, one methyl of the quarternary nitrogen has been replaced by ethyl; its potency is decreased by a factor of 3, which is also consistent with previous studies.[1]

TABLE 3. NEOSTIGMINE ANALOGS

Compound	Structure	Rel. Potency
Neostigmine		1
3-Methylcarbamoxyphenyltrimethylammonium (norprostigmine)		1·3
3-Hydroxyphenyltrimethylammonium		0·55
Edrophonium (Tensilon)		3
Pyridostigmine		Weak receptor inhibitor

If the quaternary nitrogen is incorporated into the ring, the compound (pyridostigmine) becomes a weak receptor inhibitor. Its first effect can be noted at a concentration of 1×10^{-3} M. It acts at the synapse only; the direct spike is not affected. Its inhibition can be overcome by a sufficiently increased concentration of activator. Fig. 9 represents the dose-response curves of the compounds from Table 3. It can be seen that the maximum depolarization for neostigmine, norprostigmine and 3-hydroxyphenyltrimethylammonium is identical, and is reached by all three compounds at a concentration of 5×10^{-4} M. The activity at lower concentrations varies (see Table 3). Tensilon also produces its maximum depolarization at 5×10^{-4} M, but the steady state potential is about 15 mV higher. If the concentrations of Tensilon and neostigmine are increased to 1×10^{-2} M, the membrane repolarizes and reaches

a steady state between −60 and −70 mV (see Fig. 9). Norprostigmine and 3-hydroxyphenyltrimethylammonium have not been tested at 1×10^{-2} M.

Acetyl-β-methylcholine. Acetyl-β-methylcholine is a racemic compound, and it has been shown previously that only the D-isomer is hydrolyzed by AChE. The L-isomer acts as an inhibitor.[9] It seemed of interest to test these isomers on the electroplax. Neither the D-isomer, L-isomer, nor the racemic mixture depolarizes the membrane at a concentration as high as 1×10^{-2} M; they were tested in the presence and in the absence of physostigmine, 5×10^{-5} M. Both the D- and the L-isomer blocked the indirect spike and inhibited the effect of carbamylcholine. The D-isomer was more potent than the L-isomer; the difference was 5-fold. The lowest concentration of the D-isomer which produced inhibition of the response to carbamylcholine was $7{\cdot}5 \times 10^{-4}$ M. The L-isomer did not potentiate ACh, although it is an inhibitor of AChE.

DISCUSSION

The effects observed with low concentrations of physostigmine, which have no effect on electrical activity, are clearly due to the inhibition of AChE. Acetylcholine acts only if its hydrolysis by AChE is prevented. The maximum response to ACh is seen in the presence of physostigmine, 5×10^{-5} M. There seemed to be a quantitative relationship between the depolarizing action of ACh and the amount of physostigmine present (Figs. 1 and 2), as one would expect from a reversible competitive inhibitor of AChE. At concentrations lower than 5×10^{-5} M, a steady state is also established (Fig. 2), which is not the case with irreversible or slowly reversible inhibitors such as phospholine.[10]

The transient depolarizations caused by physostigmine, 5×10^{-5} M, which are triggered by stimulating the nerve endings, can also be explained by the inhibition of AChE. These depolarizations are apparently due to the release of ACh, which acts on the same receptors as curare, since the depolarizations are prevented by it. Acetylcholine seems to be released very close to the receptor sites, i.e. within the membrane; this would explain its effectiveness, since it is reversed rapidly by the diluting effect of the physostigmine solution bathing the innervated membrane of the electroplax. Changing the solution increases the rate of recovery. Direct (cathodal) stimulation of the conducting membrane does not cause an increased depolarization in the presence of physostigmine. This is not surprising, since it has been shown that physostigmine affects the conducting membrane only in much higher concentrations due to the structural barriers surrounding the excitable membrane in conducting structures.

The antagonism of high concentrations of physostigmine to ACh and carbamylcholine in the electroplax[8] and on the frog rectus preparation[11] has been shown before. The results reported in this paper were obtained by using a Varian ink recorder to measure the resting potential which allows one to follow the time course of depolarization and to determine steady states. Physostigmine acts on the conducting membrane, but only at concentrations higher than 5×10^{-5} M. It blocks the directly evoked action potential in the electroplax and acts on a variety of nerve fibers.[8, 12]

Acetylcholine, propionylcholine and butyrylcholine in the presence of physostigmine, 5×10^{-5} M, are almost equally potent depolarizers of the electroplax. However, butyrylcholine is hydrolyzed by AChE at a much slower rate[13] than are ACh and propionylcholine. The latter two compounds are not active without physostigmine, but butyrylcholine alone still depolarizes to the same maximum as in the presence of

physostigmine, although its potency is decreased by a factor of 41 due to its hydrolysis by acetylcholinesterase (AChe). These three compounds have the same efficacy in their action on the receptor, but the rates of hydrolysis by the enzyme differ. This further supports the assumption that the active sites of enzyme and of receptor are different. Isovalylcholine also depolarizes, but it is 24 times weaker than ACh. Since isovalylcholine does not require physostigmine for maximal effect, this decrease in potency is due to a decrease in efficacy. The same applies to hexahydrobenzoylcholine. The substitution of an aromatic ring confers local anesthetic properties on the ACh analog.[2]

Wilson and coworkers reported that methylcarbamates are more potent inhibitors of acetylcholinesterase than dimethylcarbamates.[14] It seemed of interest to test these same compounds on the electroplax. It can be seen from Table 2 and Fig. 7 that their relative depolarizing potencies on the electroplax are in the opposite direction from their inhibitory strengths on the enzyme, the same applies for neostigmine and norprostigmine. This again indicates that receptor and enzyme have different active sites. Neostigmine is less potent than its hydrolysis product, 3-hydroxyphenyltrimethylammonium, and the latter in turn is less potent than the simple ion, phenyltrimethylammonium.[15] This again is the opposite order of potency as compared with their inhibitory actions on the enzyme.

No explanation can be offered at present for the repolarization of the membrane by high concentrations of neostigmine and edrophonium.

Acetyl-β-methylcholine is a specific substrate for acetylcholinesterase; it is not hydrolyzed by other cholinesterases. Hoskin tested the D- and L-isomers on eel AChe and found that only the D-isomer is hydrolyzed, while the L-isomer is an inhibitor.[9] The fact that acetyl-β-methylcholine does not depolarize the electroplax, despite the high degree of stereospecificity on the enzyme, is another strong argument that receptor and enzyme actions are taking place on different sites.

Acknowledgements—I would like to thank Professor David Nachmansohn for his advice and guidance during the course of this work. I am also grateful to Dr. F. C. G. Hoskin for suggesting the use of the D- and L-isomers of acetyl-β-methylcholine.

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