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DIFFERENCE BETWEEN TETRACAINE AND *d*-TUBOCURARINE IN THE COMPETITION WITH CARBAMYLCHOLINE

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SUMMARY

The antagonistic action of tetracaine and tetracaine methiodide on depolarizations produced by carbamylcholine using monocellular electroplax preparation has been studied. A two-fold action for these two compounds has been demonstrated.

One action occurs at the synapse and results in competition with *d*-tubocurarine and carbamylcholine. The second action is localized at the conducting membrane and is responsible for preventing the depolarizing currents produced at the synapses by carbamylcholine from spreading along the conducting membrane. The effect of tetracaine on the conducting membrane occurs at a concentration of $3.3 \cdot 10^{-5}$ M, while $5 \cdot 10^{-5}$ M is required to demonstrate competition at the synapse. Tetracaine methiodide $1 \cdot 10^{-5}$ M produces an effect on the conducting membrane and $2 \cdot 10^{-5}$ M on the synapse. It is suggested that the effect of these two compounds on the conducting membrane is similar to the competition demonstrated at the synapse, but the competition occurs between acetylcholine, which is released within the membrane by the action of carbamylcholine at the synapse, and tetracaine.

It was also found that, while quaternary tetracaine methiodide apparently has a higher affinity for the receptor compared to tertiary tetracaine, it is a weaker blocking agent. The effects of the two compounds on the conducting membrane, however, are additive.

INTRODUCTION

The isolated single electroplax developed by SCHOFFENIELS[1] has become through recent improvements a highly sensitive, versatile and stable preparation whose responses to chemical compounds are very reproducible[2]. For a specialized cell, like the electroplax, alterations in the electrical potentials are probably a direct indication of the reaction occurring between chemical compounds affecting the elementary process and their receptor sites. The preparation is, therefore, very useful for correlating chemical reactions responsible for electrical activity of biological membranes.

Many compounds related in structure to acetylcholine have been found either to depolarize the innervated membrane or to antagonize this action. The first type has been referred to as receptor activators, and the latter as to receptor inhibitors. It was not until recently, however, that an adequate demonstration of competition

was made between these two groups of compounds. These authors[3] were able to show that the inhibitors were capable of repolarizing the membrane following depolarization, and this repolarization occurred in the presence of the same concentration of activator that previously depolarized. In the case of *d*-tubocurarine and either carbamylcholine or acetylcholine this type of depolarization–repolarization could be carried through several sequences by raising the concentration slightly of one compound followed by an increase in the other. Further studies on the competition between *d*-tubocurarine and carbamylcholine permitted an estimate of the apparent dissociation constant of *d*-tubocurarine[2]. A direct competition for sites at the synapse between the activator and inhibitor seems to adequately explain these results. With tetracaine, however, a second depolarization following a repolarization was difficult to obtain, and it appeared desirable to investigate these effects further.

In the present experiments the action of tetracaine was studied with improved techniques allowing certain differences between tetracaine and *d*-tubocurarine to be explained. Also a description of the action of tetracaine has been made which is more detailed than was previously possible.

METHODS

Single electroplax from the Organ of Sachs of Electrophorus were prepared according to the techniques developed by SCHOFFENIELS[1].

The recording arrangement was the same as those used previously[2]. This method allows an accurate determination of the changes in resting potential. Cells whose action potentials, either direct or indirect, were less than 100 mV were not used.

Single impalements, using 3 M KCl filled microelectrodes of 7–10 MΩ resistance, were maintained for as long a period of time as the change in resting potential gave a smooth continuous curve. No damage could ever be attributed to the insertion of the microelectrode for prolonged periods of time, although occasionally variations in resting potentials did occur in various parts of the cell, especially in depolarized ones. If, for some reason, a reimpalement was necessary, successive impalements were made, but the experiment was continued only if good agreement was obtained between several impalements. At the end of an experiment several impalements were also made to insure that no alteration in the recording apparatus had occurred during the experiment.

The volume of the chamber facing the innervated side of the electroplax was 1 ml. When the solution was changed, this chamber was washed rapidly with 5–15 ml of the desired solution. It was determined that continuous washing did not alter the final equilibrium potential or the rate at which it was achieved, but when a solution was to be used for a long period of time the solution was periodically replaced with 5–10 ml of fresh solution.

RESULTS

Equilibrium potential as used in this paper refers to the steady potential to which a given concentration of carbamylcholine depolarizes the conducting membrane. For convenience the equilibrium potential will be referred to as a positive number. As has been shown previously, an equilibrium potential can be determined for various concentrations of carbamylcholine, and it is relatively constant from cell to cell[2].

Fig. 1 shows a group of curves obtained with increasing concentrations of carbamylcholine in the presence of various concentrations of tetracaine. The electroplax was incubated in the tetracaine solution for 30 min prior to the application of carbamylcholine, and the carbamylcholine was added to the solution maintaining the tetracaine at its initial concentration. Each point on the graph is the mean equilibrium potential obtained on at least 4 cells and from a minimum of 2 eels. The mean initial resting potential is —72.7 mV (65.0–87.2), but since the absolute values of the depolarization are independent of the initial resting potential, and because of the variation in this potential, it is not included in this figure.

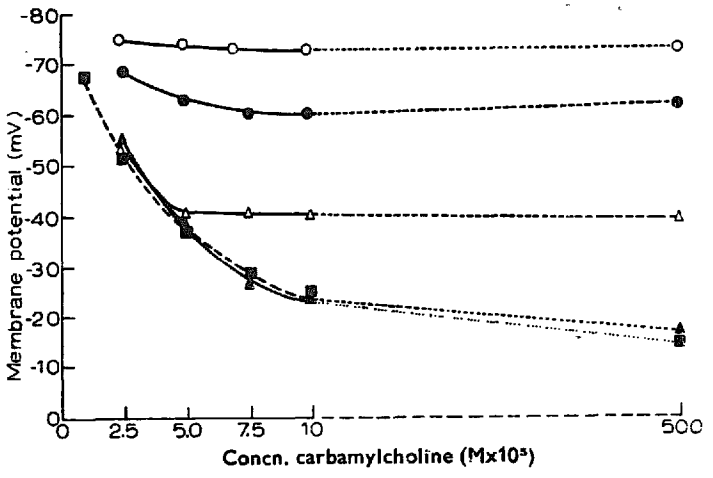

Fig. 1. The effect of tetracaine on carbamylcholine depolarizations using the isolated single electroplax. The points (■---■) of the lowest curve represent the mean equilibrium potential for the concentrations of carbamylcholine given by the abscissa. The remaining curves show the degree of depolarizations of carbamylcholine in the presence of constant concentrations ▲—▲, $1 \cdot 10^{-5}$ M; △—△, $3.3 \cdot 10^{-5}$ M; ●---●, $5 \cdot 10^{-5}$ M; O—O, $1 \cdot 10^{-4}$ M of tetracaine as the inhibitor.

At the lowest effective concentration of tetracaine ($3.3 \cdot 10^{-5}$ M) the maximum possible depolarization results in an equilibrium potential of about 40 mV. Using concentrations of carbamylcholine less than $5 \cdot 10^{-5}$ M, which gives an equilibrium potential greater than 40 mV, no competition is apparent.

The antagonistic effect of tetracaine on carbamylcholine depolarizations thus differs markedly from that of curare which shows competition at all levels of depolarization[2]. The difference in behavior of curare and tetracaine may be attributed, as will be seen later, to the absence of an antagonistic action of $3.3 \cdot 10^{-5}$ M tetracaine at the synapse. With $5 \cdot 10^{-5}$ M tetracaine a competitive effect on the synapse cannot be ascertained on the basis of these data, but it does takes place as shown in the experiments described below.

Fig. 2 was obtained with the same method as Fig. 1 except that the quaternary tetracaine methiodide was used in place of the tertiary tetracaine. The antagonistic action of tetracaine methiodide to carbamylcholine depolarizations is about twice as potent as its tertiary analogue. The figure also indicates that tetracaine methiodide ($2 \cdot 10^{-5}$ M) competes with concentrations of carbamylcholine less than $7.5 \cdot 10^{-5}$ M. The mean initial resting potential of the cells recorded in this figure is —77.6 mV (67.5–89.0).

The concentration of the tertiary tetracaine that was found to block both the direct and indirect action potentials consistently within 30 min was $3.3 \cdot 10^{-5}$ M;

$1 \cdot 10^{-5}$ M was found to block occasionally. Sometimes, as has been previously reported[4], the indirect spike was blocked shortly before the direct, but this does not conclusively demonstrate that the action on the synaptic membrane preceded that on the conducting membrane. It should be remembered that a block of the indirect

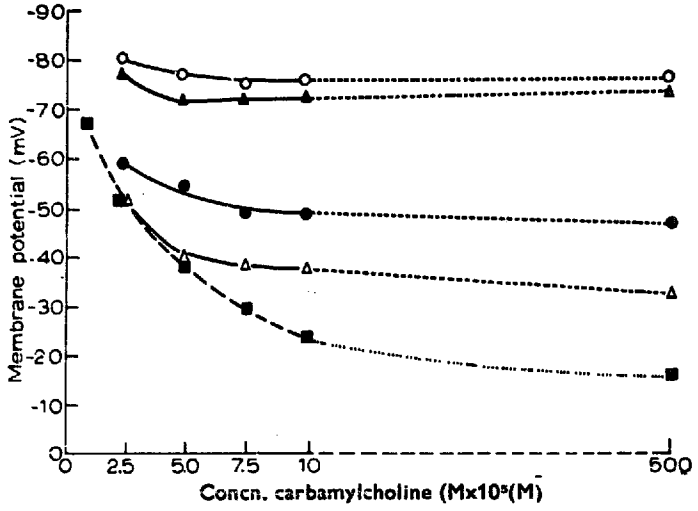

Fig. 2. The effect of tetracaine methiodide on depolarizations. The lowest curve (■---■) represents the depolarizations produced by carbamylcholine alone, and the remaining curves, the depolarizations in the presence of tetracaine methiodide: △—△, $1 \cdot 10^{-5}$ M; ●—●, $2 \cdot 10^{-5}$ M; ▲—▲, $3.3 \cdot 10^{-5}$ M; O—O, $1 \cdot 10^{-4}$ M.

spike may be due to compounds that act at the synaptic membranes exclusively such as curare, or on the nerve fibers innervating the conducting membrane such as tetracaine might be expected to do.

While the antagonistic action of tetracaine methiodide to carbamylcholine depolarization is stronger than that of the tertiary tetracaine, the latter is about 3 times

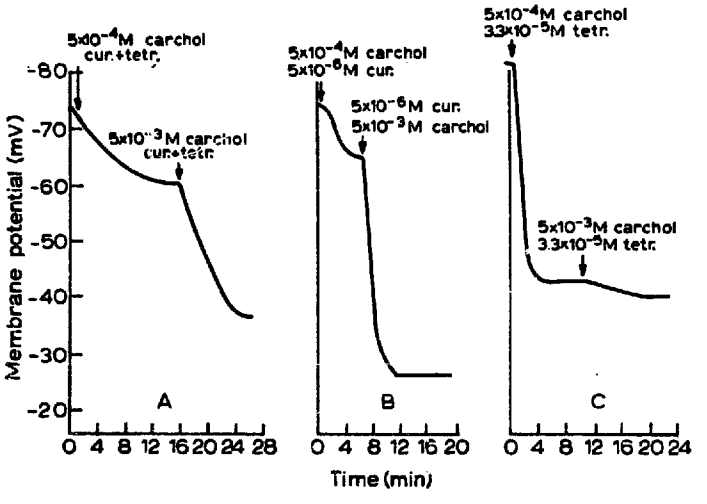

Fig. 3. The independent effects of tetracaine and curare. A. An electroplax was preincubated in $5 \cdot 10^{-6}$ M curare (cur.) for 30 min, and then the preincubation was continued in the curare plus $3.3 \cdot 10^{-5}$ M tetracaine (tetr.) for another 30 min. The solutions indicated at the arrows were applied after the preincubation was completed. (Carchol is carbamylcholine.) B. A second electroplax was preincubated in $5 \cdot 10^{-6}$ M curare; following the preincubation the solutions indicated at the arrows were applied. C. A third electroplax was preincubated in $3.3 \cdot 10^{-5}$ M tetracaine; following the preincubation the solutions indicated at the arrows were applied.

as potent in blocking the direct and indirect responses. A concentration of $1 \cdot 10^{-4}$ M tetracaine methiodide does not block either the indirect or direct response, however, both electrical responses are blocked if $5 \cdot 10^{-5}$ M carbamylcholine is added, even though no significant depolarization occurs.

In the experiment shown in Fig. 3, Section A, tetracaine was added after the electroplax had been preincubated for 30 min in curare; the preincubation was continued for 30 min in curare plus tetracaine. In the experiment of Section B the cell was preincubated for 30 min in curare only and in Section C only in tetracaine. The curves shown in this figure were obtained on three different cells. Although the first depolarization shown in A and B are not exactly the same, the equilibrium potentials do fall within the ranges recorded for successive depolarizations by carbamylcholine in the presence of curare. The same holds true for the second depolarization shown in A and C.

The first depolarization in Section A is shown to reach an equilibrium potential that is approximately equal to the first of B, although the equilibrium is reached at a lower rate, but both are markedly less pronounced than the first depolarization of C. The potential reached during the second depolarization is about the same in A and C, but different from B. Since the first depolarization with curare plus tetracaine and with curare alone (A and B) are similar, and the second depolarization with curare plus tetracaine and with tetracaine alone (A and C) are similar, it is apparent that no synergistic action takes place at this concentration of tetracaine.

These observations suggest the possibility that the absence of an effect of tetracaine in the presence of curare during the first depolarization of A may be due to the inability of tetracaine at this concentration to compete with carbamylcholine as was also indicated in Fig. 1. During the second depolarization of Sections A and C the presence of tetracaine in the conducting membrane may antagonize the action of acetylcholine, which is released in conducting membrane by the depolarization of the synapses with carbamylcholine. The second depolarization in Section B on the other hand reflects the direct competition between carbamylcholine and curare taking place exclusively at the synapse.

The possibility that the effect of $3.3 \cdot 10^{-5}$ M tetracaine described in Fig. 3 is not at the synapse but on the conducting membrane was tested in Fig. 4. Carbamylcholine ($5 \cdot 10^{-4}$ M) depolarizes the membrane to approx. —15 mV, if $5 \cdot 10^{-6}$ M curare is added

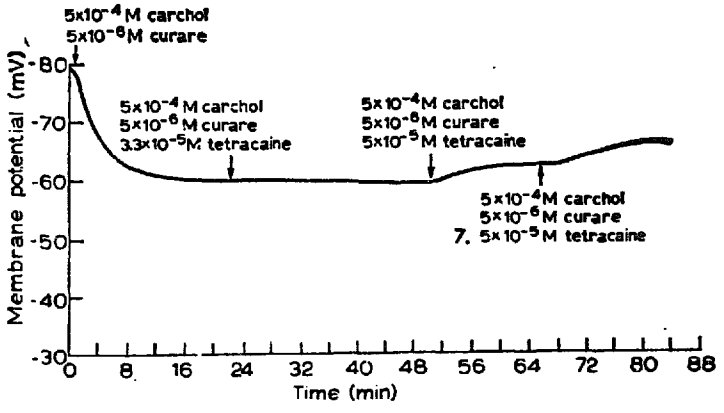

Fig. 4. The ability of tetracaine to repolarize the conducting membrane extrasynaptically. The solutions indicated at the arrows were applied after the electroplax had been preincubated in $5 \cdot 10^{-6}$ M curare for 30 min. (Carchol is carbamylcholine).

the membrane depolarizes to only —60 mV (see Fig. 3B also). The smaller depolarization is due to curare-occupying sites which carbamylcholine would have occupied in the absence of curare. Tetracaine ($3.3 \cdot 10^{-5}$ M) plus $5 \cdot 10^{-4}$ M carbamylcholine depolarizes the membrane to —40 mV. Since this is a weaker depolarization than carbamylcholine would have produced alone, it might be expected that tetracaine is preventing the depolarization of carbamylcholine by combining with a certain number of receptor sites. Since a large portion of the functional receptor sites must be occupied when the membrane is partially depolarized in carbamylcholine and curare, the addition of $3.3 \cdot 10^{-5}$ M tetracaine should replace some of the carbamylcholine from the synaptic receptors which would result in a repolarization. Fig. 4 shows that a repolarization does not occur with $3.3 \cdot 10^{-5}$ M tetracaine. If, however, the concentration of tetracaine is raised to $5 \cdot 10^{-5}$ M or $7.5 \cdot 10^{-5}$ M, a repolarization of the membrane takes place, but only to the same level as with tetracaine alone in the absence of curare (see Fig. 1). This experiment shows that $3.3 \cdot 10^{-5}$ M tetracaine has no effect on the synapse. With $5 \cdot 10^{-5}$ M tetracaine, however, competition which would result in a repolarization of less than 2 mV, or less than 7 mV with $7.5 \cdot 10^{-5}$ M, might be obscured by the stronger effect of tetracaine on the conducting membrane. It is clear, nevertheless that the repolarization is principally due to the effect of tetracaine on the conducting membrane.

As was previously pointed out in Fig. 2, the curve for $2 \cdot 10^{-5}$ M tetracaine methiodide is different from the carbamylcholine curve before maximum depolarization is obtained. This indicates the possibility of a competition at the synapse. Since a total depolarization of approx. 25 mV is possible at this concentration, it appeared likely that synergism between tetracaine methiodide and curare could be demonstrated. In Fig. 5 the cell is depolarized to 65 mV with $5 \cdot 10^{-4}$ carbamylcholine in the presence of $5 \cdot 10^{-6}$ curare. When the equilibrium potential is reached, $2 \cdot 10^{-5}$ tetracaine methiodide is added, and a repolarization takes place. Without curare the same concentration of carbamylcholine and tetracaine methiodide depolarize the membrane to about —46 mV. Thus, it is apparent that in these concentrations, tetracaine methiodide displaces carbamylcholine from some of the receptor sites. If, however, the concentration of carbamylcholine is increased to $5 \cdot 10^{-4}$ M, the same maximum depolarization is obtained as if curare was not present. This shows that

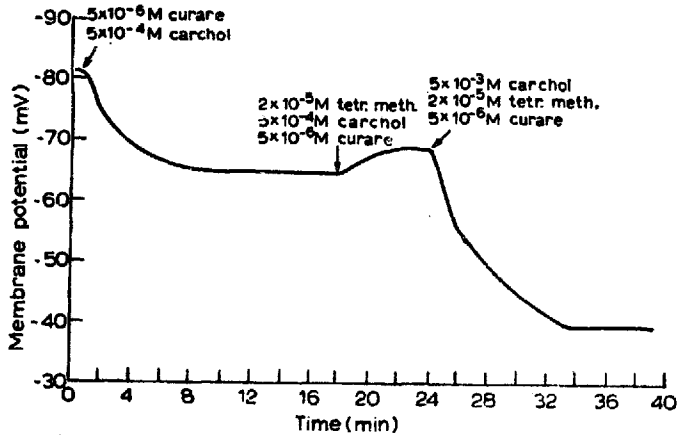

Fig. 5. Competition between tetracaine methiodide (tetr. meth.) and carbamylcholine (carchol) for synaptic receptor sites. The solutions indicated at the arrows were applied after the electroplax had been preincubated in $5 \cdot 10^{-6}$ M curare for 30 min.

another action of tetracaine methiodide takes place at the conducting membrane where it does not compete with carbamylcholine directly, but with acetylcholine released in the membrane by the depolarizing action of carbamylcholine on the synapse. These results clearly show that the tetracaine methiodide acts on two sites: at the synapse where it antagonizes carbamylcholine directly, and at the conducting membrane where carbamylcholine is not active except through the spread of depolarizing currents originating at the depolarized synapses.

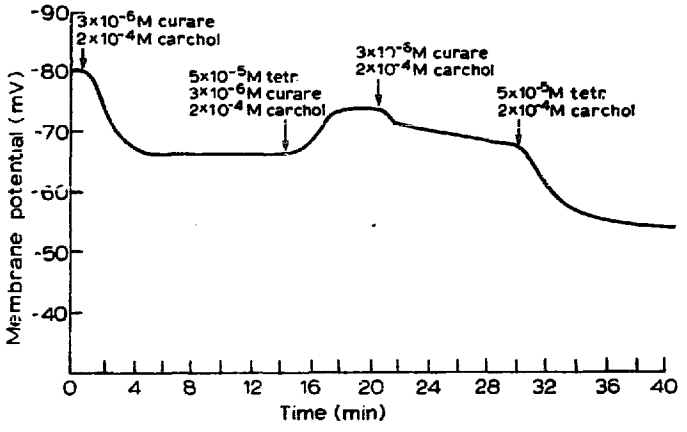

Fig. 6. Competition between tetracaine (tetr.) and carbamylcholine(carchol). The solutions indicated at the arrows were applied after the electropax had been preincubated in $3 \cdot 10^{-6}$ M curare for 30 min.

Fig. 6 shows the same type of experiment as described in Fig. 5 except that $5 \cdot 10^{-5}$ M tertiary tetracaine was used. Fig. 1 indicated that $5 \cdot 10^{-5}$ M of tetracaine is the only concentration where sufficient depolarization occurs, and also where the curve is apparently different from the carbamylcholine curve before the maximum possible depolarization is obtained. Fig. 6 shows that at this concentration competition between curare, tetracaine and carbamylcholine does occur at the synapse. In order to test whether the repolarization in Fig. 6 was real, tetracaine was removed, and the membrane potential returned to the same level reached before the addition of tetracaine. When curare was removed the depolarization increased to the same maximum predictable from Fig. 1.

The competition between tetracaine and carbamylcholine can only be observed with rather small depolarizations, and is therefore not readily demonstrable. In six

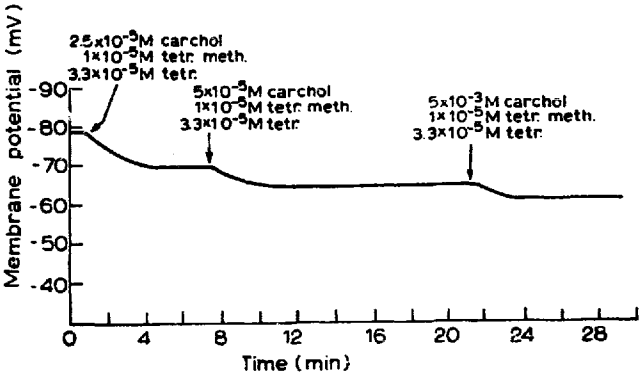

Fig. 7. The additive effect of tetracaine (tetr.) and tetracaine methiodide (tetr. meth.). The solutions indicated at the arrows were applied after the electroplax had been preincubated in $1 \cdot 10^{-5}$ M tetracaine methiodide plus $3.3 \cdot 10^{-5}$ M tetracaine. (Carchol is carbamylcholine).

out of ten experiments, results similar to those shown in Fig. 6 were obtained. In the other four no repolarization occurred when the tetracaine was added to the curare and carbamylcholine, but because of the smallness of the effects, overlapping of the potentials may easily obscure the repolarizations.

Fig. 7 demonstrates that the effects of tetracaine and tetracaine methiodide are additive. The cell was incubated for 30 min in $1 \cdot 10^{-5}$ M tetracaine methiodide plus $3.3 \cdot 10^{-5}$ M tetracaine. Since the effect of tetracaine methiodide on the carbamylcholine depolarization is approx. 2 times more potent than tetracaine, these two concentrations are equivalent to about $5 \cdot 10^{-5}$ M tetracaine. If the equilibrium potentials of Fig. 7 are compared to those of Fig. 1 for $5 \cdot 10^{-5}$ M tetracaine, it may be seen that the summation obtained corresponds exactly to that expected on the basis of Figs. 1 and 2.

It has been reported that several local anesthetics hyperpolarize the membrane of nerve fibers[5,6]. Concentrations of $3.3 \cdot 10^{-5}$ M and $1 \cdot 10^{-3}$ M tetracaine or tetracaine methiodide did not hyperpolarize the electroplax membrane in periods of time lasting up to 30 min.

DISCUSSION

A method has been described[2] which permits the use of the membrane potential to determine the apparent dissociation constants of carbamylcholine and curare on the receptor, and also to demonstrate their competitive action. When an attempt was made to apply the same method for determining the dissociation constant of tetracaine, a marked difference became apparent between curare and tetracaine. Since both compounds are receptor inhibitors, such a difference was at first unexpected.

Several differences between tetracaine and curare were, however, already known. Since tetracaine blocks the direct action potential, and curare does not, tetracaine must penetrate the conducting membrane. In contrast, the action of compounds such as curare and carbamylcholine is limited to the synapses. Unlike curare, carbamylcholine blocks both the direct and indirect action potentials because the depolarization, initiated at the synapses, spreads throughout the innervated membrane.

NACHMANSOHN has suggested that tertiary nitrogen derivatives, analogous in structure to acetylcholine, such as tetracaine, may effect the conducting membrane due to their higher lipid solubility in contrast to the absence of such action of the lipid-insoluble quaternary nitrogen derivates such as curare. He and his associates have accumulated considerable evidence in support of this assumption[7-10]. One might have expected, therefore, differences in the competitive action between tetracaine and carbamylcholine, compared with that of curare as was observed in the present study.

The major effect of tetracaine and tetracaine methiodide (Figs. 1 and 2) is the decrease of the maximum depolarization that high concentrations of carbamylcholine ($5 \cdot 10^{-3}$ M) can produce. The maximum possible depolarization gradually decreases with increasing concentrations of inhibitor until a sufficiently high concentration ($1 \cdot 10^{-4}$ M) prevents any significant depolarization by carbamylcholine. Repolarization by tetracaine previously reported[3] and shown in Fig. 4 only occurs when the membrane potential is below the maximum depolarization possible in the concentration of tetracaine added to the solution which is to demonstrate repolarization. Once the cell is repolarized, it cannot be depolarized by increasing the concentration

of carbamylcholine, because the electroplax cannot be depolarized further. The repolarization of the conducting membrane occurs because of the action of tetracaine on the conducting membrane even though the synapses possibly remain depolarized.

The dissociation constants of the receptor protein with small molecules such as curare, carbamylcholine and tetracaine are at present not known. The "apparent" dissociation constants for curare and carbamylcholine measured on the intact electroplax give only a relative indication of the binding strength, since in an organized structure the reaction is a complex process depending on a number of factors, such as for instance, permeability and reaction with other extracellular and membraneous cell constituents by electrostatic and London dispersion forces. Even the reaction with the acetylcholine receptor protein may occur not only on the active sites where the affinity would be expected to be high, but to some extent with other groups of the protein although with a much lower affinity. From the data presented it is apparent that at the synapse tetracaine in $3.3 \cdot 10^{-5}$ M is unable to compete with carbamylcholine. Only when the concentration of tetracaine is raised to $5 \cdot 10^{-5}$ M can competition at the synapse between carbamylcholine and tetracaine be demonstrated; at this concentration also a synergistic action between curare and tetracaine is observed. In contrast, the competitive effect of tetracaine on the conducting membrane is observed at $3.3 \cdot 10^{-5}$ M. This concentration is the same which blocks the electrical response, both direct and indirect, in the absence of carbamylcholine. These data indicate that tetracaine competes effectively with acetylcholine released within the membrane at a concentration 30% lower than that required to show competition with the action of carbamylcholine acting on the receptor in the synapse. The action of carbamylcholine on the synapse should not affect the potency of tetracaine on the conducting membrane because there, even in the presence of carbamylcholine in the external solution, the inhibitor competes only with acetylcholine released internally.

While the demonstration of an effect on the conducting membrane prior to an effect on the synaptic membrane is clear, a complete evaluation is difficult nevertheless, because of several unknowns in the process. Acetylcholine released is extremely unstable since it will be rapidly hydrolyzed in contrast to carbamylcholine. The quantities of acetylcholine released in the conducting membrane may not necessarily be quite the same, although they are most likely of a similar order of magnitude. The synaptic membrane may differ slightly in their sensitivity from that of the conducting membrane. Even pre- and post-synaptic membranes vary in their sensitivity to externally applied compounds. Curare blocks the electrical activity of the nerve terminal at a lower concentration than that required for the block of the endplate potential generated in the postsynaptic membrane[11]. Whether these differences are due to differences in receptor concentration or to other factors is at present an open question.

The number of excess receptor sites available to acetylcholine in the conducting membrane and the nature of the release of acetylcholine within the membrane may be responsible for several of the results described in this paper. In $3.3 \cdot 10^{-5}$ M tetracaine for example, since no antagonism between acetylcholine and tetracaine is measured until the maximum depolarization is obtained, it would appear that a sufficient number of receptor sites are left unoccupied by tetracaine to allow acetylcholine to produce normal depolarizations. At about —40 mV, however, if a further

depolarization occurs at the synapse, it is apparently necessary for acetylcholine to replace some tetracaine in order to produce the increased depolarization. Since acetylcholine cannot overcome the effect of tetracaine, acetylcholine is probably not released in sufficient high quantities to replace the tetracaine. Increasing concentrations of tetracaine would then be expected to decrease the maximum possible depolarization that can be produced by carbamylcholine, as shown in Fig. 1.

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