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MOLECULAR FORCES ACTING BETWEEN AMMONIUM IONS AND ACETYLCHOLINE RECEPTOR

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Abstract—The reaction between the acetylcholine (ACh)-receptor and quaternary ammonium ions, containing no carbonyl group, have been studied on the isolated electroplax preparation and the results compared with those previously obtained with the same ions on the activity of ACh-esterase.

The depolarizing strength of *n*-alkyltrimethylammonium ions does not follow the same order as that of the inhibitory strength on the enzyme. Inhibition of the enzyme has been reported to increase with the addition of each —CH_2 group. The activity of these ions on the ACh-receptor does not increase in a linear fashion. The order of increasing strength of depolarization is: butyltrimethylammonium > pentyl- > hexyl- > propyl- > methyl- > ethyl- ⋙ hydrogen. Since the activation of the receptor presumably involves conformational changes, a greater specification of chemical forces is required for activation of the receptor than for the inhibition of the enzyme.

Of the aryltrimethylammonium ions studied, the phenyl- and benzyltrimethylammonium ions were the most active. Their depolarizing activity was about equal to that of pentyltrimethylammonium ion. The substitution of groups on carbon 3 of the ring reduced the activity more than substitution on carbon 4. All the groups substituted on carbon 3 or 4 reduced activity, and the results were not related to the expected change in dipole moment, group size, or to the electron withdrawing or contributing properties of the substituted group. Again, marked differences were found between ACh-esterase and ACh-receptor. The order of increasing strength of depolarization is: benzyl- > phenyl- > 4-methylphenyl- > 3-methylphenyl- > 3-chlorophenyl- > 3-hydroxyphenyl- > 3-methoxyphenyl.

Tetra-*n*-alkylammonium ions, except tetramethylammonium, do not activate the ACh-receptor. They reduce the maximum depolarization of the synapses resulting from the action of an activator. The relative potencies for the antagonism observed at the synapses correspond to the inhibitory strength reported on the ACh-esterase. The order of increasing inhibition is as follows: tetrapentyl- > tetrapropyl- > tetrabutyl > tetraethyl.

These observations offer additional support for the assumption that the active site of the ACh-receptor, in spite of some similarities, differs in important features from that of ACh-esterase.

MANY YEARS ago, Nachmansohn suggested that the change in permeability of excitable membranes during electrical activity was due to the ability of acetylcholine (ACh) to cause a conformational change in a protein (ACh-receptor).[1,2] It was postulated that the change in conformation could initiate the reactions resulting in a change in the ionic permeabilities of excitable membranes. Hydrolysis of ACh by ACh-esterase restores the resting state of the receptor and thereby the barrier to ion movements. Some indirect evidence has been found for conformational changes in the two enzymes associated with the formation and hydrolysis of ACh, i.e. ACh-esterase and choline

acetylase.[3,4] The ACh-receptor has not been isolated;[5] therefore, a study of the chemical properties of the receptor is dependent upon measurements of the electrical properties of intact excitable membranes. Observations made on these membranes support the assumption that some properties of the receptor are comparable to those of the enzyme ACh-esterase,[6,7] although some others are distinctly different.[8,9]

The use of intact cells for the purpose of determining properties of a macromolecule results in several complications which cannot be evaluated at the present time. The importance of cellular and subcellular organization for the activity of enzymes and proteins has, however, become increasingly apparent in recent years. The studies on respiratory enzymes and oxidative phosphorylation[10] (mitochondria) and on fatty acid synthesis[11] may be mentioned as illustrations. In order to understand the role of a macromolecule in cellular function, studies on the proteins in organized structures, e.g. membranes, may under certain conditions provide important information even if the protein is not yet available in solution.

The isolated single electroplax preparation from the electric eel, *Electrophorus electricus*, has many unique features making these cells suitable for the study of the properties of the ACh-receptor system in the intact excitable membrane.[2,12] The electroplax are biochemically and physiologically highly specialized for the generation of electrical potentials. This tissue is poor in proteins (3 per cent) and rich in water (93 per cent),[13] but the proteins associated with electrical activity are relatively abundant; therefore, the tissue is valuable for the study of these proteins. Furthermore, highly sensitive and dependable methods have been developed for monitoring the interaction of small molecules with the intact ACh-receptor system of isolated electroplax.[7,9,14–16]

Because of the simple structure and poor chemical reactivity of *n*-alkyl and aryl quaternary ammonium ions and the many data available on the physico-chemical binding properties of these ions to purified ACh-esterase,[17,18] it is desirable to investigate the behavior of these ions with the ACh-receptor. There have been many studies made on the activity of these quaternary ammonium ions on synaptic membranes; however, in these studies muscular contraction has usually been the means of measuring the effect of the ion.[19] It has been recognized for a long time that this procedure gave only indirect results, since the direct effect of these ions is to alter the electrical properties of the excitable membranes. For technical reasons, however, the changes in membrane potential obtained with the use of microelectrodes have only been made in a few cases and under conditions not as well defined as with the electroplax. No complete study has been made attempting to correlate small changes in the chemical structure of these ions, which contain no ester linkage, with the effects on the membrane potentials.

The effects of a series of *n*-alkyl and simple aryl ammonium ions have been studied in the present work. The response of the membrane to these ions was measured with an internal microelectrode. Furthermore, assuming that the ACh-esterase and ACh-receptor possess some molecular features making them relatively specific for ACh, one might assume that at least some similar molecular forces exist in the active centers of both macromolecules. A comparison between the reactions of the quaternary ammonium ions with ACh-esterase and those with the ACh-receptor has been made in an attempt to elucidate the similarities and differences between the active centers of ACh-esterase and ACh-receptor.

METHODS

The methods used for dissecting and mounting the isolated single electroplax have been described in detail previously.[12] The method used for measuring the activity of ACh-receptor activators and inhibitors has also been described[14] and may be summarized as follows. The action of an "activator", a ligand which depolarizes the synaptic membranes, is determined by measuring the steady state membrane potential in various concentrations of the activator. After the application of an activator to the isolated electroplax, an initial fast decrease in the electrical potential across the innervated membrane occurs. After a few minutes, however, a new steady state potential is established and no further change in membrane potential occurs. The new steady state potential is dependent only upon the concentration of the activator and the potential is constant among cells from the same or from different specimens. The average steady state potential is determined on a number of cells and these potentials are used as a measure of the interaction between the ACh-receptor and activator. Competition for receptor sites by an activator and inhibitor is measured by comparing the steady state potentials in solutions of activator alone and in solutions containing both activator and inhibitor.

It has also been found that the standard error of the mean for each steady state membrane potential is about the same, i.e. about $\pm$ 3–5 mV, when 3 or more cells are used for determining the mean.[14] Therefore, the standard errors have not been included in the present data, but comparable standard errors were calculated. Membrane potential differences of less than $\pm$ 5 mV should not be considered as significant. Furthermore, when an average membrane potential is used, the value has been determined on between 3 and 10 cells from at least 2 different eels.

The change in membrane potential is monitored continuously by a glass microelectrode (10 MΩ) filled with 3 M KCl solution and inserted into the interior of the isolated electroplax. The electrode is left in place throughout the experimental procedures. If, however, the electrode becomes dislodged, reinsertions are possible.

Several derivations[20] have been made of the equation for the dissociation constant of receptor inhibitors. The equation used in the present work is:

$$K_I = \frac{[I]\,[A]}{[A'] - [A]} \tag{1}$$

K_I is the dissociation constant of the inhibitor; I, the concentration of inhibitor used; $[A]$, the concentration of activator used in the absence of I; and $[A']$, the concentration of activator in the solution containing $[I]$ required to give the same membrane potential as $[A]$. Cells were incubated in the inhibitor solutions for 30 min prior to the addition of the activator.

Tetraethylammonium bromide, tetra-*n*-propylammonium bromide, tetra-*n*-butylammonium bromide, tetra-*n*-pentylammonium iodide and phenyltrimethylammonium chloride were purchased from Eastman Organic Chemicals. Tetraethylammonium iodide, ethyltrimethylammonium iodide, propyltrimethylammonium bromide, butyltrimethylammonium bromide, pentyltrimethylammonium bromide, hexyltrimethylammonium iodide, benzyltrimethylammonium bromide, trimethylamine bromide and the 3-hydroxy-, 3-methoxy-, 3-methyl- and 4-methylphenyltrimethylammonium iodides were gifts from Dr. S. Ginsburg.

RESULTS

1. n-*Alkyltrimethylammonium ions.* Fig. 1 shows the effect of altering the alkyl chain length of a homologous series of *n*-alkyltrimethylammonium ions on the ability of the ion to depolarize the isolated electroplax. Steady state membrane potentials (— mV) for each concentration of ammonium ion were determined and the results from a minimum of 3 cells were averaged and plotted vs. the log concentration of ion. The length of the alkyl chain is indicated by the letter "*n*", which is equal to the number of carbon atoms in the formula, $(CH_3)_3N^+C_nH_{2n+1}$.

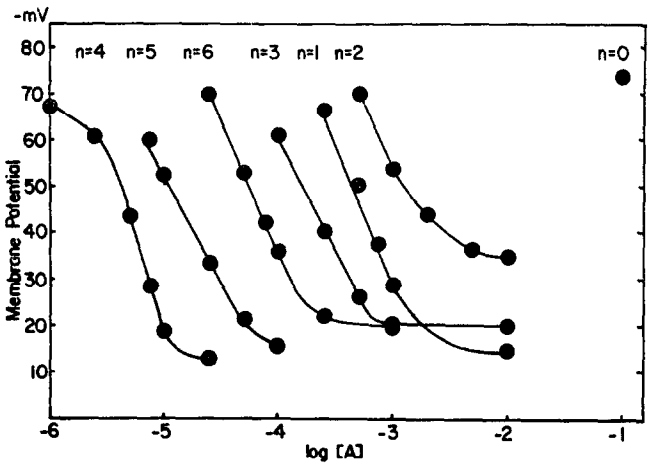

FIG. 1. The effects of altering the length of the *n*-alkyl chain of a homologous series of alkyltrimethylammonium ions on their depolarizing action studied on the isolated electroplax. The ordinate represents the membrane potential of the innervated side and the abscissa, log [*A*], the log of the concentration of each ammonium ion. The structure of this series is $(CH_3)_3N^+C_nH_{2n+1}$; the "*n*" for each ammonium ion is indicated; $n = 0$ is $(CH_3)_3N^+H$. Each point represents the mean membrane potential obtained on a minimum of 3 cells, and for the points falling on the linear portion of the curve at least 4 cells were used. The usual limits of the range in measurements were less than ± 5 mV of the mean. As stated in the Methods section, the accuracy of the membrane potential measurements applies to all experimental procedures and a similar number of experiments was made to determine each mean value. The mean initial resting potentials (E_0) were; n = 0, — 79 mV; $n = 1$, — 72 mV; $n = 2$, — 72 mV; $n = 3$, — 83 mV; $n = 4$, — 71 mV; $n = 5$, — 73 mV; $n = 6$, — 72 mV.

With the exception of ethyltrimethylammonium ion, a lengthening of the chain increases the depolarizing activity of the ion until the chain contains five carbon atoms. Pentyltrimethylammonium and hexyltrimethylammonium are weaker activators than butyltrimethylammonium. The most potent activator is butyltrimethylammonium and the least potent is trimethylammonium.

The maximum depolarizations, except for trimethylammonium and ethyltrimethylammonium, are about the same as for carbamylcholine and ACh, i.e. the minimum steady state potential is about — 15 mV. The maximum depolarizations by hexyltrimethylammonium and propyltrimethylammonium may also be slightly less than that by the other ions, but measurement of differences in the maximum depolarization becomes very difficult when the membrane potential is less than — 20 mV. Potential changes, below — 20 mV, in response to an increase in the concentration of the

activator are very small. If, however, the curves as plotted in Fig. 1 become non-near at membrane potentials larger than — 20 mV, the maximum depolarization is definitely reduced.

Bergman and Segal[17] have measured the inhibitory strength of a series of *n*-alkyl-trimethylammonium ions on ACh-esterase isolated from electric tissue of *Electrophorus*. In contrast to the reaction with the receptor, increase of the chain length from one carbon through seven carbons increases the strength of these compounds as inhibitors of ACh-esterase. Fig. 2 shows the comparison of the data obtained on

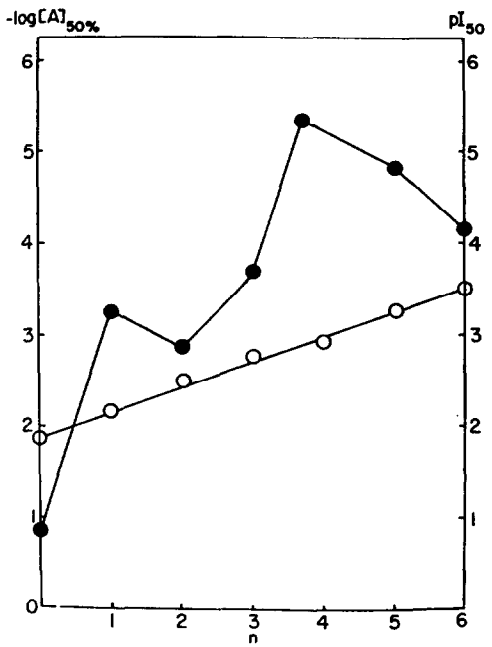

FIG. 2. The effect of increasing the n-alkyl group of a homogous series of *n*-alkyltrimethylammonium ions on their ability to inhibit ACh-esterase compared to that on the depolarizing action of the isolated electroplax. The — log of the molar concentration of each ammonium required to depolarize the innervated membrane to — 45 mV, or approximately 50 per cent of the total maximum depolarization, is plotted on the left ordinate as — log $[A]_{50\%}$ and is represented by the closed circles (●). On the right ordinate is plotted pI_{50} (— log of the molar concentration of each ion required to produce 50 per cent inhibition of the ACh-esterase), which is represented by the open circles (○). The latter data are taken from Bergmann and Segal.[17] On the ordinate is plotted "n", the number of carbon atoms in the ion whose empirical formula is $(CH_3)_3N^+C_nH_{2n+1}$.

ACh-esterase and ACh-receptor. The relative depolarizing strengths of the ammonium ions were determined by selecting the concentration of the ion required to lower the membrane potential to — 45 mV. The concentration for trimethylammonium had to be obtained by extrapolating from the point given in Fig. 1 to — 45 mV by using a slope of the linear portion of the curves obtained with the other ions. As will be discussed later, it is impossible to relate directly these differences between inhibition of ACh-esterase and the activation of the receptor as a function of binding. The fact, however, that the two curves in Fig. 2 are radically different is difficult to reconcile with the assumption that the receptor and esterase possess identical active centers.

2. *Aryltrimethylammonium ions.* By comparing the data given in Fig. 1 for butyltrimethylammonium with similar data obtained with ACh,[21] it can be shown that these two compounds are about equally active in causing a depolarization of the electroplax. This observation suggests that the ACh-receptor may not contain another site comparable to the esteratic site of ACh-esterase. Therefore, in an effort to test for the presence or absence of an esteratic site in the receptor, several analogs of phenyltrimethylammonium were used. These compounds were selected for two reasons. First, they are simple analogs of the quaternary ammonium ions already discussed and second, Wilson and Quan[18] have found them useful in studying the active center of ACh-esterase.

By using phenyltrimethylammonium as the reference compound, 3-hydroxyphenyltrimethylammonium was found to be 120 times more effective an inhibitor of ACh-esterase; 3-methoxyphenyltrimethylammonium was 5 times more effective; and benzyltrimethylammonium was about half as effective. The addition of the methyl group in the 3 or 4 position of phenyltrimethylammonium did not markedly alter the inhibitory strength. The large increase in the inhibitory strength of 3-hydroxyphenyltrimethylammonium was attributed to a hydrogen bond which was oriented in such a manner that the bond probably was formed with the esteratic site of the enzyme.

The depolarizing activity of this series of compounds is shown in Fig. 3. Bartels[22] had determined the activity of 3-hydroxyphenyltrimethylammonium on electroplax;

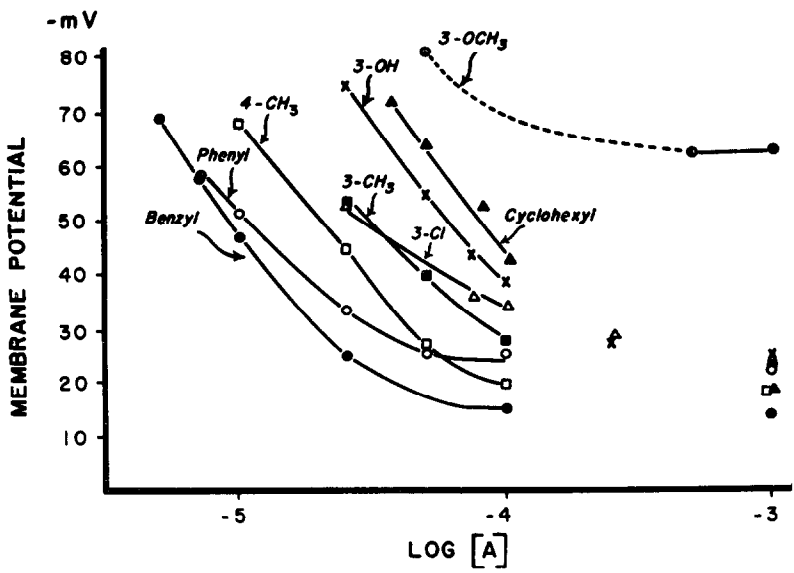

FIG. 3. The depolarizing activity of a series of aryltrimethylammonium ions. The innervated membrane potential (— mV) is plotted on the ordinate, and the log of the molar concentration of the ions used is plotted on the abscissa (log [A]). Mean steady state potentials which result from the action of benzyltrimethylammonium (●), E_0 = — 74 mV; of phenyltrimethylammonium (○), E_0 = — 72mV; of 4-methylphenyltrimethylammonium (□), E_0 = — 75 mV; of 3-chlorophenyltrimethylammonium (△), E_0 = — 75 mV; of 3-methylphenyltrimethylammonium (■), E_0 = — 75 mV; of 3-hydroxyphenyltrimethylammonium (×), E_0 = — 78 mV; of 3-methoxyphenyltrimethylammonium (⊙), E_0 = — 81 mV; of cyclohexyltrimethylammonium (▲), E_0 = — 71 mV. E_0 is initial membrane potential.

comparable observations were made in the present study. Compared to phenyltrimethylammonium, 3-hydroxyphenyltrimethylammonium is one-fifth as active as an activator, whereas 3-methoxyphenyltrimethylammonium is a much weaker activator, approximately 65 times less effective than phenyltrimethylammonium. Also, the maximum depolarization induced by these compounds is measurably different. The maximum depolarization obtained with 3-hydroxyphenyltrimethylammonium and phenyltrimethylammonium was about the same, but for 3-methoxyphenyltrimethylammonium the maximum depolarization was reduced very markedly. Obviously, the addition of the hydroxyl group did not make phenyltrimethylammonium a stronger receptor activator, as might have been expected if the strength of ACh as a receptor activator was enhanced by the binding at a site comparable to the esteratic site of ACh-esterase.

In addition to the data shown in Fig. 3, it was also noted that a phenomenon, sometimes referred to as a "desensitization" of the receptor,[23] occurred with several of these quaternary nitrogen compounds; after a rapid depolarization with high concentrations of activators, a transient repolarization of the membrane potential occurred, although the concentration of the activator was maintained. This observation was made with tetramethylammonium, 3-hydroxyphenyltrimethylammonium and phenyltrimethylammonium. No quantitative measurements were attempted, but it did appear that 3-hydroxyphenyltrimethylammonium resulted in the most pronounced repolarization and tetramethylammonium in the least. It is clear, however, that the repolarization following a rapid depolarization is not dependent upon a compound containing a linkage susceptible to hydrolysis, e.g. ACh and carbamylcholine.

As with the *n*-alkyltrimethylammonium compounds, it is difficult to relate the measurements of activation of the receptor and inhibition of the esterase. With these small modifications in structure, however, an orderly relationship between the two measurements would be expected if the receptor and esterase were identical. The order of increasing activation of the receptor is benzyl- > phenyl- > 4-methyl- > 3-methyl > 3-hydroxy- > 3-methoxy-, whereas on the enzyme the order of increasing inhibition is 3-hydroxy- $\gg$ 3-methoxy- > 3-methyl- > 4-methyl- > phenyl- > benzyl- > trimethylammonium.[18] Although, in general, stronger inhibitors of ACh-esterase tend to be weaker activators of the ACh-receptor, no real correlation exists; the methoxy derivative, for instance, is a much weaker receptor activator than the hydroxy, while the latter is a much greater inhibitor of ACh-esterase. Also, the quantitative differences between these two analogs are far from being the same on the esterase and the receptor; 3-hydroxyphenyltrimethylammonium is 120 times better an inhibitor of the esterase than the 3-methoxy, but as an activator of the receptor the 3-methoxy is only about 3 times weaker.

As seen in Fig. 3, phenyltrimethylammonium and benzyltrimethylammonium ions have virtually the same potencies. The addition of the $-CH_2$ group to phenyltrimethylammonium to form the benzyl derivative results in no significant alteration in activity, in contrast to the addition of a $-CH_2$ in the series of alkyltrimethylammonium compounds. Also, the addition of a phenyl group does not prevent these ring structures from depolarizing the synaptic membrane. Furthermore, the ring does not appear to alter significantly the depolarizing activity of phenyltrimethylammonium compared to the simple alkyl compounds, if one examines the molar

volume of the phenyl and pentyl groups as measured by molar refraction.[24] Phenyltrimethylammonium has almost the same activity in equimolar concentrations with *n*-pentyltrimethylammonium (see Figs. 1 and 3), and molar refraction measurements on the phenyl and pentyl groups are nearly equal. In this regard it should also be noted that hexyltrimethylammonium and cyclohexyltrimethylammonium have about the same potency.

Another compound to be mentioned is *N*-methylpyridinium. It is not a derivative of the aryltrimethylammonium ions, but it does have structural similarities. *N*-methylpyridinium possesses a quaternary nitrogen, but only one methyl is free. The other carbon atoms on the nitrogen are incorporated into the structure of the ring. As measured on the ACh-esterase, *N*-methylpyridinium has a K_I approximately half that of phenyltrimethylammonium.[25] The K_I values for *N*-methylpyridinium and benzyltrimethylammonium are nearly the same. On the electroplax, 10^{-3} M *N*-methylpyridinium was found to be without effect as an activator or as an inhibitor.

3. *Dissociation constants of* d-*tubocurarine determined with alkyl- and aryltrimethylammonium ions.* From the derivation of the equation used for determining the dissociation constants of *d*-tubocurarine (see equation 1 in Methods), it should be noted that the constant is independent of the activator used. It has been shown on frog muscle that the use of ACh and carbamylcholine as activators results in the same K_I for *d*-tubocurarine.[20]

In an effort to prove that the quaternary ammonium ions studied in the two previous sections are similar to other depolarizing compounds, e.g. ACh and carbamylcholine, and to support the validity of equation 1, the dissociation constant for *d*-tubocurarine was determined by using tetramethylammonium and benzyltrimethylammonium as activators. The same K_I (2×10^{-7} M) was obtained for *d*-tubocurarine by using either activator and this value agrees with the figure previously determined with carbamylcholine.[14] The data for this determination are shown in Fig. 4. Equal responses required for the solution of equation 1 may be obtained by comparing the linear portions of the curves for the activator alone with the activator plus 1×10^{-6} M *d*-tubocurarine. From Fig. 4 it can be seen that the presence of *d*-tubocurarine does not alter the slope of the curves. The slopes of both curves in the presence of *d*-tubocurarine are very close to — 60 mV for a 10-fold change in activator concentration.

4. *Tetra*-n-*alkylammonium ions.* A homologous series of tetra-*n*-alkylammonium ions was studied and, as reported by other investigators using the neuromuscular junction,[19, 26] it was found that these compounds do not depolarize the electroplax. These ions inhibit the depolarization of the membrane by receptor activators, e.g. carbamylcholine. The results of these experiments are shown in Fig. 5.

The experiments represented in Fig. 5 were done in a manner identical to those shown in Fig. 4, where *d*-tubocurarine was used as the inhibitor. Comparing Figs. 4 and 5 shows that the presence of *d*-tubocurarine does not alter the maximum depolarization of an activator, but the tetra-*n*-alkylammonium ions reduce the maximum depolarization. Tetraethylammonium at 1×10^{-3} M did not reduce the maximum depolarization, and a K_I of $2{\cdot}5 \times 10^{-4}$ M was calculated from the data. An increase in the concentration of tetramethylammonium to 1×10^{-2} M, however, markedly reduced the maximum depolarization, as seen in Fig. 5, but had no effect on the duration or amplitude of the direct action potential.

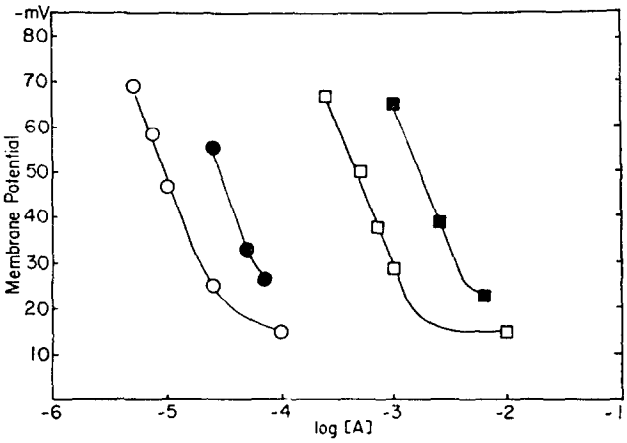

FIG. 4. Determination of the K_I of *d*-tubocurarine (curare) by using two different quaternary ammonium ions as activators. The innervated membrane potential (— mV) is plotted on the ordinate and the log of the molar concentration of the two ammonium ions is plotted on the abscissa (log [A]). Steady state potentials which result from the action of benzyltrimethylammonium in the absence (○) and in the presence (●) of 1×10^{-6} M curare; of tetramethylammonium, in the absence (□) and in the presence (■) of 1×10^{-6} M curare. Curare shifts the curves for the activator toward a higher concentration of activator. The displacement of the activator curves due to curare is approximately constant for the two activators studied. The K_I calculated for curare on the basis of the data presented is 2×10^{-7} M. This value is equal to that obtained with the use of carbamylcholine.[14]

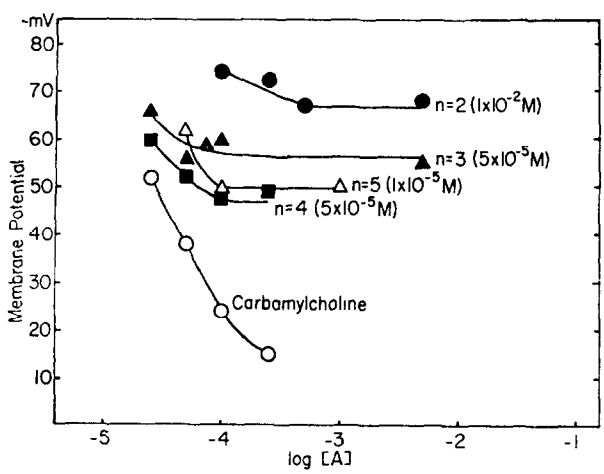

FIG. 5. The antagonistic effects of a homologous series of tetra-n-alkylammonium ions, $(C_nH_{2n+1})_4N^+$, on the depolarizations produced by carbamylcholine: tetraethylammonium, $n = 2$ (●); tetrapropylammonium, $n = 3$ (▲); tetrabutylammonium, $n = 4$ (■); tetrapentylammonium, $n = 5$ (△). The curve for the activity of carbamylcholine is shown for reference (○). Cells were incubated in the concentration of alkylammonium ion for 30 min before carbamylcholine was added to the solution. Each point represents the mean steady state potential which results from the action of carbamylcholine in the presence of the tetraalkylammonium ion indicated. As previously stated (see Methods), when mean potential measurements are used, each point represents data from at least 3 cells and has about the same standard error of about ± 5 mV.

When a reduction of the membrane potential is prevented by high concentrations of the tetraalkylammonium ions, the addition of an activator did not block the direct action potential, as has been reported previously.[27] A sample experiment demonstrating this observation is shown in Fig. 6. This experiment was done with

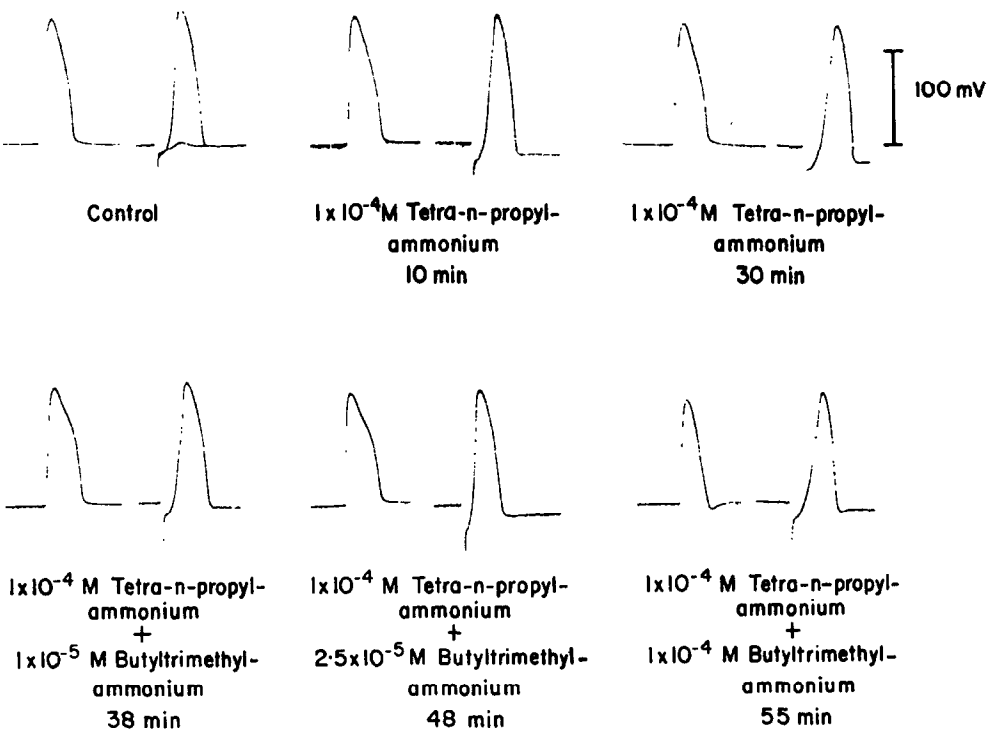

FIG. 6. The effect of butyltrimethylammonium (an activator) on the direct and indirect action potentials in the presence of 1×10^{-4} M tetrapropylammonium (an inhibitor) shows that both compounds act at the level of the ACh-excitable membrane. The direct action potential is located to the left of each pair of action potentials, and the indirect action potential, to the right of each pair. After the control potentials were recorded at 0 time, 1×10^{-4} M tetrapropylammonium was added and the cell was incubated in this solution for 30 min. At 33 min, 1×10^{-5} M butyltrimethylammonium plus 1×10^{-4} M tetrapropylammonium was added to the bath and the potentials were recorded 5 min later. At 43 min, $2{\cdot}5 \times 10^{-5}$ M butyltrimethylammonium plus 1×10^{-4} M tetrapropylammonium was added and potentials were recorded 5 min later. The concentration of butyltrimethylammonium was increased to 1×10^{-4} M at 49 min, while maintaining the tetrapropylammonium at 1×10^{-4} M, and 6 min later the potentials were recorded again. The action potentials were recorded with external electrodes. A calibration signal of 100 mV is indicated.

1×10^{-4} M tetra-*n*-propylammonium as the inhibitor and butyltrimethylammonium as the activator. The lowest concentration of butyltrimethylammonium used in Fig. 6, 1×10^{-5} M, is sufficient to block the action potential in the absence of tetrapropylammonium. In the presence of 1×10^{-4} M tetrapropylammonium, 1×10^{-4} M butyltrimethylammonium did not block the direct action potential. The data shown in Fig. 5 indicate that 1×10^{-4} M tetrapropylammonium would be expected to prevent butyltrimethylammonium from depolarizing the membrane potential below — 60 mV. The resting membrane potential of the cell used in Fig. 6 was measured, and it was

found that the potential did not begin to decrease until the concentration of butyltrimethylammonium was 1×10^{-4} M.

Podleski and Bartels[28] have shown that very high concentrations of activator are unable to reduce the membrane potential in the presence of tetracaine and tetracaine methiodide. The addition of low concentrations of activator, however, blocks both action potentials, even though no significant depolarization occurs. This effect is especially prominent with tetracaine methiodide. The tetraalkylammonium ions are different, since they do not block the direct action potential in the concentrations studied, and the addition of an activator in the presence of these ions does not block the direct action potential.

A comparison of the results shown in Fig. 6 with those obtained on ACh-esterase, a purified preparation obtained from electric eel tissue, shows several similarities. The maximum velocity of enzymatic hydrolysis of ACh is reduced in the presence of the tetraalkylammonium ions,[25, 29] just as the maximum depolarization is reduced. Furthermore, the sequence of the association constants obtained on the enzyme is as follows: tetra-*n*-propyl > tetra-*n*-butyl > tetraethyl.[30] The order of increasing ability to antagonize the action of an activator is obtained from the data of Fig. 5, and the order is found to be identical to that found on the enzyme. Similar studies have been made on erythrocyte cholinesterase;[29] the dissociation constants calculated for the tetraalkylammonium ions on the two enzymes do not agree; however, a decrease in the maximum velocity on the erythrocyte enzyme similar to that on the electric tissue enzyme was shown. The K_I calculated for tetraethylammonium mentioned above, $2{\cdot}5 \times 10^{-4}$ M, is the same as that measured on electric tissue ACh-esterase.

DISCUSSION

Several difficulties are encountered when the attempt is made to correlate the depolarizing activity of receptor activators with their affinity for theACh-receptor. The maximum depolarizations, for instance, are not the same for all the ions studied. The reason for these differences is not clear and the understanding of the differences is important for the analysis of the curves shown in Figs. 1 and 3 in physiochemical terms.[14, 16, 31] The maximum depolarization may not represent a saturation of receptor sites,[16, 31] and whether for some activators the depolarization is limited by other features (for example those determining the ionic permeabilities of the synaptic membranes[32]) is not clear. Also the role of the process referred to as "desensitization" in determining the maximum depolarization remains to be solved. Because of these difficulties, no effort was made to calculate the dissociation constants for the activators. It should be noted, however, that when muscular contraction has been used to study the alkylammonium ions[33,34] equal maximum contractions were obtained with ions which in the present study showed unequal maximum depolarizations. Such results are to be expected, since the coupling of contraction to membrane potential is indirect and frequently not linear.[35]

In reference to receptor desensitization, several observations should be emphasized. The steady state potentials used in Figs. 1 and 3 were steady for periods of time lasting 30 min and longer; no spontaneous repolarization was ever measured. Furthermore, an increase in the concentration of quaternary ammonium ion resulted in the same further depolarization, whether the initial concentration had been applied for 5 min

or 30 min. The steady state potentials appear to be independent of time after the first 5 min, which is the approximate time required for the steady state to be achieved. While these experiments do not indicate that a significant degree of desensitization occurred with concentrations of the activator smaller than those required for maximum depolarization, similar experiments performed with frog sartorius muscle do show desensitization at low concentrations of carbamylcholine.[36] Low concentrations of activators were selected when the activities of the ions were compared (for example, see Fig. 2), since desensitization has not been demonstrated on the electroplax except in high concentrations of activator. The complicating role of desensitization is thereby minimized.

At the present time the innervated membrane of the electroplax is viewed as containing areas which are sensitive to externally applied ACh and its congeners coupled to insensitive membrane. The voltage–current relationship of the insensitive membrane has been determined and two resistance states have been shown to exist in the potential range of − 80 to − 10 mV.[37] Upon depolarization of the innervated membrane by about 30 mV, there occurs a transition in the membrane resistance from a low to a higher level. Since the voltage–current relationship of the electroplax membrane is not linear, it is necessary to compare the potency of the various depolarizing ligands at identical membrane potential values. Since the dose–response curves are nearly parallel to one another, however, any potential will yield about the same results. Furthermore, it has recently been observed that in 25 mM CsCl, where the voltage–current relationship is nearly linear and the resting resistance of the membrane is increased 50 per cent,[37] no marked change in the dose–response curve of carbamylcholine occurs.[38]

The need for obtaining precise quantitative data about the activity of homologous activators, in spite of the difficulties, is useful and pertinent for the understanding of the mechanism of receptor activation and the nature of the receptor's active center. The molecular forces acting between the receptor and its inhibitors are often not specific; inhibitors may frequently be large molecules with several unsaturated rings and hydrophobic chains. Therefore, the interaction of inhibitors with the active center may be influenced by groups removed from the active center. The active center of the receptor must be studied with activators as well as with inhibitors, since the former are usually small molecules with few reactive and more specific groups. In general, the same principle applies to enzyme substrate interaction: the specifications of substrate structure are usually much greater than for those of inhibitors.

In recent years many attempts have been made to analyze the molecular forces responsible for the interaction of small ions with macromolecules.[39] Often these attempts have been unsatisfactory due to the many unknown characteristics of macromolecules. Fortunately for the study of the ACh-receptor, a closely associated protein, ACh-esterase, has been studied under about the same conditions, i.e. ionic strength, pH and temperature, with notable success,[17, 18, 25] and the simple ammonium ions used in the present work raise the question as to whether any known physicochemical parameter varies in a manner which could account for the observations made in Figs. 1 and 3.

Recently, observations have been made which showed that an unionized hydroxyl group about 5 Å from the quaternary nitrogen of hydroxymethylquinolinium ions decreased the K_I of these ions as receptor inhibitors.[7] Apparently a distance of 5 Å

for $\overset{+}{N} \rightarrow OH$ is favorable for hydrogen bond formation of receptor as well as the enzyme protein. This observation indicates similarity of binding between the active sites of ACh-esterase and ACh-receptor, which is not apparent from the study of activators. Correlations between strong inhibitors of ACh-esterase and weak activators of the ACh-receptor may prove to be important: possibly a stronger binding may weaken the potency of the receptor activator.

The role of hydrophobic binding forces is generally recognized as important in the intramolecular forces of proteins,[40, 41] and a role in the binding of substrates and inhibitors to enzymes has been suggested.[42, 43] Mautner *et al.*[44] have suggested that hydrophobic forces may be important in the binding of activators to the receptor. The variations in potency of the ammonium ions cannot be explained by hydrophobic forces alone. The effects of the addition of $-CH_2$ groups to a series of amino acids show that the solubility ratio of alcohol to water increases linearly with each $-CH_2$ group added,[45] and the solubilities of methane, ethane, *n*-propane and *n*-butane in water are known to decrease in this order.[46] It is difficult therefore to attribute the decrease in activity observed with ethyltrimethylammonium to a lower hydrophobicity of the ethyl group. The linear curve shown in Fig. 2 for the inhibition of ACh-esterase is more likely due to hydrophobic or dispersion forces and these results agree with the observations made on several other enzymes.[42, 43]

There is also no simple correlation between receptor activation and hydrophobicity of the aryl ions. In two studies the hydrophobicity of the phenyl group has been reported to be equivalent to that of the *n*-propyl group.[41, 42] Since the activity of phenyltrimethylammonium was about equivalent to that of pentyltrimethylammonium and was therefore ten times more active than propyltrimethylammonium, no correlation between depolarizing activity and hydrophobicity is indicated. Also, the known solubilities of the benzene derivatives in water are as follows: benzene > toluene > chlorobenzene > anisole.[47] The series does not correspond to the depolarizing activity of their quatenary nitrogen analogs.

The molecular models (Fisher, Hirschfelder and Taylor) of the *n*-alkyltrimethylammonium ions reveal that the ethyl and *n*-propyl groups are severely limited in the configurations they can assume. The alkyl chains which contain more than three carbon atoms can vary in length from a fully extended chain to several contracted configurations, although there may be large energy barriers to the contracted forms.[48]

Compared to the simple alkyl chains, which can assume different conformations, the phenyl group is a rigid planar structure of known dimensions. The molecular models of the phenyltrimethylammonium and benzyltrimethylammonium ions reveal little difference in the distance between the nitrogen and carbon number 4. The orientation of the nitrogen in the latter is limited to above or below the plane of the ring, whereas in phenyltrimethylammonium the nitrogen is in the same plane as the ring. The similar activities of these two cations on the electroplax would seem to be accounted for by these structural similarities.

The alkyl chain, *n*-pentyl, can assume a configuration virtually the same as that of the phenyl in both length and bulk. Since pentyltrimethylammonium, phenyltrimethylammonium and benzyltrimethylammonium have identical potencies, it seems possible that the pentyl chain may be active in the contracted form. The hexyltrimethylammonium is longer than the phenyl group even when fully contracted and

hexyl is a weaker activator. The hexyl- and cyclohexyltrimethylammonium ions were found to have the same potency.

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