*SIMILARITIES BETWEEN ACTIVE SITES OF ACETYLCHOLINE RECEPTOR AND ACETYLCHOLINESTERASE TESTED WITH QUINOLINIUM IONS**

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Evidence has accumulated in favor of the assumption that the action of acetylcholine (ACh) is essential for the control of permeability changes taking place in excitable membranes during electrical activity. ACh apparently induces conformational changes in a protein of the membrane, the ACh receptor; the resulting shift of charge initiates a series of reactions permitting accelerated ion flow; ACh is rapidly inactivated by ACh esterase and the resting condition is restored.[1-3] While this theory was essentially based on the demonstration that potent, specific, and competitive inhibitors of either ACh esterase or ACh receptor block electrical activity in various types of excitable membranes, in recent years the actions of ACh and related compounds have been found on axonal membranes comparable to those previously described on synaptic membranes, and a release of ACh has been demonstrated from the axonal membrane under conditions comparable to those prevailing at nerve endings.[4-6] Many of the differences of biophysical manifestations and of pharmacological effects found between axons and junctions have been explained in terms of cellular structure and organization rather than on the basis of a different role of ACh in the permeability changes of synaptic and axonal membranes.

In view of the crucial role of these two proteins in the activity of excitable membranes, ACh receptor and ACh esterase, knowledge of their properties and the mechanism of their action is essential. In an early phase of the investigations,[7,8] a highly purified form of ACh esterase was obtained in solution from electric tissue. This enzyme preparation has been used over many years for studies of the molecular forces in the active site of the enzyme.[9, 10] The information obtained has provided important insight into many problems associated with the function of excitable membranes.

The ACh receptor has not been isolated. However, about 10 years ago a monocellular electroplax preparation of *Electrophorus electricus* was developed by Schoffeniels[11-13] and greatly improved over the years by Higman, Podleski, and Bartels.[14, 15] Many extraordinary features of this cell, such as its large size and the rectangular shape of the excitable membrane, permit a quantitative and remarkably reliable evaluation of the reactions of ACh and related compounds with the ACh receptor. Small changes in the structure of small molecules effect in a specific way marked changes in the reaction with the receptor, and the reaction with the receptor can be monitored by sensitive biophysical methods, i.e., the recording of various electrical parameters of the excitable membrane. While 15 years ago protein and enzyme chemists would not have considered as pertinent studies on proteins or other macromolecules unless carried out on pure material, recent developments have documented the importance of structure and organization for the functions of proteins in subcellular fractions. The mitochondrial membrane has attracted much interest

among biochemists and electron microscopists (see, e.g., refs. 16–18). Also, the brilliant experiments of Ephraim Katchalski and his associates[19, 20] on the effects of the microenvironment of enzymes on their reactivity, e.g., the reactivity in a "Faraday cage," have a particular bearing on the study of proteins within a membrane.

Dissociation constants between the ACh receptor and a variety of compounds have been determined using the monocellular electroplax preparation. Differences as well as similarities between active sites of the receptor and esterase have been found. Recently, the critical role of SH and S-S groups in the sequence of the permeability change of the excitable membrane induced by ACh has been demonstrated, thus supporting the view of a crucial role of proteins in this process.[21] Among the types of compounds studied with both proteins may be mentioned a series of benzoquinonium and ambenonium derivatives,[22] a series of aryl and alkyl ammonium ions,[23] and a series of sulfur and selenium isologs of ACh, choline, and related compounds synthesized by Mautner and his associates (see, e.g., refs. 24, 25). On the basis of these studies it has become apparent that a negative group, referred to as "anionic site" and attracting the quaternary nitrogen group by Coulombic and van der Waals' forces, is present and has similar properties in the esterase and receptor proteins. In contrast, another site referred to as the "esteratic site" in ACh esterase, in which a covalent bond is formed between the oxygen of serine and the carbonylcarbon of an ester, also plays an essential role in the ACh receptor. Data are accumulating indicating that at this site the molecular forces are distinctly different in the two proteins.

This paper reports observations made with quinolinium derivatives which show that the active site of the ACh receptor may contain an atom which forms a hydrogen bond with receptor inhibitors, if the spatial relationship between the positively charged nitrogen and a hydroxyl group is about 5 A. This hydrogen bond formation is similar to that observed with inhibitors at the active site of the ACh esterase with a similar ($\overset{+}{N} \rightarrow OH$) distance.

The hydrolysis of a series of isomeric 1-methyl-acetoxyquinolinium iodides catalyzed by ACh esterase has recently been analyzed by Prince using a newly developed and highly sensitive spectrophotometric method.[26] When isomers were compared with an acetoxy group in the 5-, 6-, 7-, and 8-position, it was found that the K_m of 1-methyl-7-acetoxyquinolinium was the lowest. Estimating the nitrogen to carbonyl-carbon atoms ($\overset{+}{N} \rightarrow C = O$) distances in the four isomers with the aid of Fisher-Hirschfelder and Dreiding models, Prince found that the distance to the 7-position was about 4.8–5.9 A, which is, among the isomers tested, closest to the distance of about 5 A separating, according to previous estimates, the anionic binding site and the atom to which the carbonyl-carbon is bound to the enzyme. This distance seemed to be a more important factor for the enzymic hydrolysis at pH 7 than the electrophilic properties of the carbonyl-carbon atom, although comparison between 5- and 7-derivatives showed that the most favorable K_m value is found with the compound having the most electrophilic carbonyl-carbon atom, i.e., the 7-derivative.

The 1-methyl-acetoxyquinolinium iodides are rapidly hydrolyzed when applied to the electroplax, but the hydrolysis products are receptor inhibitors. A study of a series of isomeric 1-methyl-hydroxyquinolinium iodides as receptor inhibitors

offers the possibility of testing the question whether a hydrogen bond is formed between the receptor and the inhibitor, and also the influence of the $\overset{+}{N} \rightarrow OH$ distance on the inhibitory strength. Previous studies on the inhibitory strength of a series of isomeric hydroxyphenyltrimethylammonium ions on ACh esterase had revealed that 3-hydroxyphenyltrimethylammonium is about 120 times stronger than phenyltrimethylammonium. This is equivalent to a decrease of about 3 kcal per mole in the free energy of binding. Such a large effect strongly supports the assumption that a hydrogen bond formation is involved. The binding of the 3-methoxy derivative is 26 times poorer than of the 3-hydroxy compound. The phenolic hydroxyl group apparently forms a hydrogen bond with the same atom in the esteratic site that forms a covalent link with the carbonyl-carbon of the dimethylcarbamate which is the strongest of the enzyme inhibitors.[27] This atom, as is generally accepted today, is the nucleophilic oxygen of serine. In the 2- or 4-position, hydroxyl groups increased the binding only slightly in respect to the reference compound; the same is true for the 3-methylphenyltrimethylammonium ion. In 3-hydroxyphenyltrimethylammonium the $\overset{+}{N} \rightarrow OH$ distance is about 5 A. Tested on the electroplax, phenyltrimethylammonium proved to be a receptor activator, i.e., it depolarized the cell. Presence of the hydroxyl group in the 3-position decreased the potency.[23] However, reactions of the protein molecule with an activator, leading presumably to a conformational change, are not comparable to those with an inhibitor reacting with the active site but not inducing further changes. It appeared desirable to test an inhibitor with a proper $\overset{+}{N} \rightarrow OH$ distance.

The hydroxyl group of 1-methyl-7-hydroxyquinolinium is located at a distance of about 5 A from the quaternary nitrogen and appears to have a similar geometry as the 3-hydroxyphenyltrimethylammonium. If the molecular groups in the active site of the receptor are similar to those in the esteratic site, a hydrogen bond formation might be expected.

K_I's of the quinolinium compounds were determined in the following way. The K_I was calculated by comparing the effects of carbamylcholine plus quinolinium with the effects of carbamylcholine alone.[14] The excitable membrane of the electroplax was depolarized with 5×10^{-5} M carbamylcholine. The potency of the inhibitory strength of 5×10^{-4} M of the quinolinium ion was then determined by testing the degree of repolarization in the presence of 5×10^{-5} M carbamylcholine. In various experiments it was determined that the inhibition of the carbamylcholine effect was competitive, i.e., it was ascertained that the maximum carbamylcholine effect was not altered by quinolinium and the K_I's were the same whether quinolinium was added prior to carbamylcholine, or vice versa.

As may be seen in Table 1, at pH 6.9 the K_I of the 1-methyl-7-hydroxyquinolinium is 11 times lower than the K_I of 1-methylquinolinium, and the 7-hydroxy compound has the lowest K_I of the isomers tested. In order to determine which form of the hydroxyl group, ionized or un-ionized, was the most active inhibitor, the pH of the external solution was altered. Changing the pH of the solution from 5.9 to 8.8 reduced the repolarization produced by 1-methyl-7-hydroxyquinolinium, whereas changes in pH had no measurable effect on the action of 1-methylquino-

TABLE 1

Compound	pK$_a$	Repolarization, mv	K_I (10^4 M)
1-Methylquinolinium			
pH 6.9		11.1 ± 3.4 (6)	9.3
" 8.8*		10.4 ± 2.3 (4)	9.3
" 8.8†		11.4 ± 4.9 (4)	9.3
7-OH Quinolinium	5.9		
pH 5.9		33.3 ± 1.8 (3)	0.56
" 6.9		29.6 ± 3.5 (4)	0.83
" 8.8*		15.4 ± 2.4 (3)	6.4
" 8.8†		5.7 ± 2.7 (7)	20
3-OH Quinolinium		9.4 ± 3.3 (4)	9.3
5-OH "	6.1	9.1 ± 1.7 (5)	9.3
6-OH "	7.2	11.0 ± 3.2 (4)	9.3
8-OH "	7.0	14.0 ± 1.9 (4)	6.4
6-CH_3 Quinolinium		21.0 ± 3.6 (5)	1.8
7-CH_3 "		21.7 ± 1.3 (4)	1.8

K_I's of a series of quinolinium derivatives that act as inhibitors of acetylcholine receptor. The inhibitory strength is determined with the monocellular electroplax preparation of *Electrophorus* by measuring the degree of repolarization of a cell partially depolarized with carbamylcholine. The standard error of the mean for the mv repolarization is indicated and the number in the parentheses represents the number of experiments. The pK$_a$'s are taken from ref. 26.
* Concentration of Tris buffer 1.5 mM.
† Concentration of Tris buffer 15 mM.

linium. Since the pK$_a$ of the 7-hydroxyl is 5.9,[26] the most active form is the un-ionized hydroxyl group. Assuming that the un-ionized forms of all the hydroxyquinolinium isomers are also better inhibitors than the ionized forms, the K_I listed in Table 1 for the 5, 6, and 8 hydroxy compounds are underestimated compared to the 7. Since the pK$_a$ of the 5, 6, and 8 hydroxyl groups are higher than the 7, at pH 6.9 where the comparison between the compounds was made, a larger percentage of the 5, 6, and 8 hydroxyl groups will be in their most active form compared to the 7. The 7-hydroxy isomer is therefore a better inhibitor relative to the 5, 6, and 8 isomers than is indicated in Table 1.

Because the pH in close vicinity of the ACh receptor cannot readily be determined in a precise way, it is impossible to calculate accurately a K_I for each of the two forms of the hydroxyl group. The marked difference in the results obtained in 1.5 mM Tris buffer compared to 15 mM may be due to the buffering capacity of the electroplax. Apparently, 1.5 mM Tris is not sufficient to maintain the pH in the vicinity of the ACh receptor at as high a level as 15 mM Tris. Neither concentration of Tris buffer affected the depolarization of carbamylcholine, nor the inhibition of 1-methylquinolinium. When the pH of the external solution was changed to 5.9, the pH in the vicinity of the ACh receptor was probably greater than 5.9. The inhibition of 7-hydroxyquinolinium was, however, greater at pH 5.9 than at pH 6.9, when both measurements were made on the same cell. The reason that this difference is not marked (see Table 1) is due to the variability between cells. The increase in the inhibitory strength at pH 5.9 supports the conclusion that the un-ionized hydroxyl group increases the inhibitory strength of the quinolinium ions more than the ionized hydroxyl group.

It is possible to estimate the dissociation constants of the ionized and un-ionized forms by assuming that in normal pH solutions of 6.9–7.0, the pH in the vicinity of the receptor is approximately 7. The data obtained in 15 mM Tris buffer at pH .88 shows that the ionized form of the 7-hydroxyl group must be very weak; at pH 6.9 the ionized form can account for less than a 5-mv repolarization. If the

total repolarization of 29.6 mv in pH 6.9 solution is ascribed to the action of the un-ionized hydroxyl group, a K_I of 0.088×10^{-4} M can be calculated for the un-ionized 1-methyl-7-hydroxyquinolinium. The undissociated 7-hydroxyl group appears to increase the inhibitory properties about 110 times compared to 1-methyl-quinolinium. This large effect, indicating a strong decrease in the free energy of binding, strongly suggests the formation of a hydrogen bond. Replacement of the hydroxyl group with a methyl reduces the inhibitory strength 20 times. The results are very similar to those found on ACh esterase using the phenyltrimethyl-ammonium ions referred to previously.

An effect of an electrophilic carbon in the quinolinium ions on the binding of these inhibitors cannot be ruled out completely. No correlation, however, can be made between pK_a and inhibitory strength. As was found in the studies of the ACh esterase-catalyzed hydrolysis of acetoxyquinolinium isomers, it appears that the spatial arrangement, namely, the intramolecular distance from the quaternary nitrogen to the hydroxyl group, is more important than the electrophilicity of the carbon atom in determining the inhibitory strength of ACh receptor inhibitors.

Summary.—Previous results obtained with inhibitors of ACh esterase had offered evidence that a quaternary nitrogen compound, having a hydroxyl group at a distance of about 5 A from the nitrogen, forms a hydrogen bond with a basic atom, probably the oxygen of serine, in the esteratic site, thereby strongly increasing the binding. When a series of isomeric 1-methyl-hydroxyquinolinium ions were tested as inhibitors of the ACh receptor using the intact electroplax, evidence for hydrogen bond formation between the receptor and 1-methyl-7-hydroxyquinolinium was found. The presence of the hydroxyl group increased the inhibitory strength of this compound about 110-fold, comparable to the effect previously observed with the corresponding enzyme inhibitor and equivalent to a decrease in the free energy of binding of about 3 kcal/mole. The hydroxyl group of 1-methyl-7-hydroxyquinolinium is also at a distance of about 5 A from the quaternary nitrogen, and the 7-hydroxyquinolinium is the best inhibitor found. The intramolecular distance thus seems to be an important factor in the reaction with the receptor, just as was previously found with the ACh esterase-catalyzed hydrolysis of acetoxyquinolinium isomers.

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