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APPARENT DISSOCIATION CONSTANTS FOR SEVERAL INHIBITORS OF ACETYLCHOLINESTERASE IN THE INTACT ELECTROPLAX OF THE ELECTRIC EEL*

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Abstract—Apparent dissociation constants were determined for *d*-tubocurarine, benzoquinonium, ambenonium, WIN 3286, and WIN 7789 as inhibitors of acetylcholinesterase (AChE) in the intact electroplax of *Electrophorus electricus*. These five compounds showed nearly the same order of potency for inhibiting AChE in intact electroplax cells as had been determined earlier using AChE purified from the electric organ of the electric eel. However, the constants were 140–510 times higher for cellular AChE than for purified AChE. The order of effectiveness of these five compounds as inhibitors of cellular AChE was completely different from the order previously determined for inhibition of the acetylcholine receptor of the intact electroplax of the electric eel. The ratio of the apparent dissociation constants determined for cellular AChE compared to the constants for the acetylcholine receptor varied from 0·16 to 3500. It is concluded that the active site of AChE is different from that of the acetylcholine receptor.

SOME investigators have suggested that the active sites of acetylcholinesterase (AChE) and the acetylcholine receptor (AChR) may be identical.[1–8] In the 1950's, Hoppe[9] and Lands *et al.*[10] developed benzoquinonium, ambenonium, and a series of their analogs. These *bis*-quaternary nitrogen compounds were found to be potent competitive inhibitors of the AChR and AChE. The large differences between the apparent dissociation constants for the complexes of these compounds with the AChR in single isolated electric eel electroplax and for the complexes with purified AChE obtained from the electric organ of the electric eel have been invoked as evidence for the non-identity of the two active sites.[11] However, the activity of purified AChE in solution may well be different from its activity when it is a membrane component. Therefore, in the experiments to be described, an attempt was made to determine the apparent dissociation constants for the reaction of these same inhibitors with AChE present in the intact electroplax of the electric eel.

The structures of the compounds tested are shown in Fig. 1. These new data should show whether or not the potencies of these inhibitors for membrane-bound AChE are different from the potencies for inhibiting purified AChE. If they are different, it is then of interest to compare them with the potencies for receptor inhibition. The conclusions should apply to the skeletal motor end-plate as well as to the electroplax,

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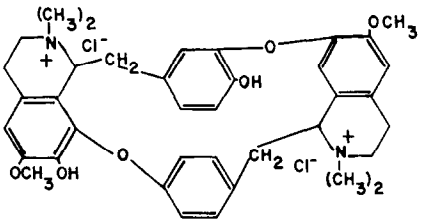
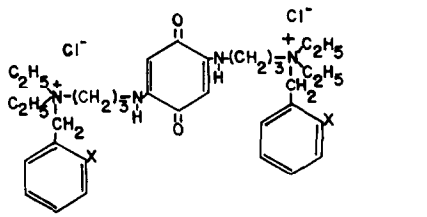
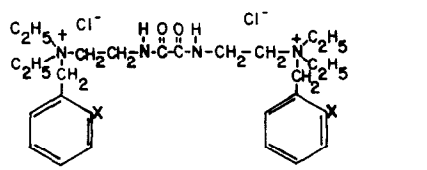


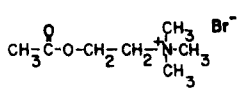
d-TUBOCURARINE



BENZOQUINONIUM (X=H) and WIN 7789 (X=Cl)



AMBENONIUM (X=Cl) and WIN 3286 (X=H)



ACETYLCHOLINE

FIG. 1. The structure of the compounds used in this investigation. In the text, *d*-tubocurarine is called curare.

since the electroplax evolved from striated muscle. In all investigations to date, the nerve-electroplax junction has been found to have pharmacological and biochemical properties nearly identical to those of the skeletal neuromuscular junction.

MATERIALS AND METHODS

Single electroplax cells were dissected by the method of Schoffeniels[12] in oxygenated electric eel physiological saline of the following composition: 160 mM NaCl, 5 mM KCl, 0·3 mM NaH_2PO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1·2 mM Na_2HPO_4, and 10 mM glucose, adjusted to pH 7·0. This is the same solution as that used in the previous investigations.[11, 13, 14] The cells used were not tested for their electrical activity, but other cells from the same eels had normal resting and action potentials. Occasionally, damaged cells were used, since it was found that small nicks in the cell did not significantly affect the AChE activity.

A modified physiological saline was used as an incubating medium in order to increase the buffering capacity. This incubating medium had the following composition: 151 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 12 mM Tris(hydroxymethyl)-aminomethane, and 10 mM glucose, adjusted to pH 7·2 to 7·4 with acetic acid.

The benzoquinonium and ambenonium compounds (chloride salts) were kindly supplied by Drs. R. O. Clinton and A. M. Lands of the Sterling–Winthrop Research Institute, Rensselaer, N.Y. The *d*-tubocurarine chloride (referred to in this paper as curare) was purchased from Sigma Chemical Co., St. Louis, Mo. The acetylcholine bromide was purchased from Eastern Organic Chemicals, Rochester, N.Y.

At the start of each experiment, two blotted cells were placed into individual 25-ml Erlenmeyer flasks. Usually four or five flasks were used per day. Each flask contained 4 ml of the incubating medium. A control containing ACh without any inhibitor was run each day. All the other flasks contained incubating medium with an inhibitor present as well as ACh. A 1-ml sample of incubating solution was removed from each flask soon after the cells were added, and the amount of ACh was determined by the colorimetric method of Hestrin.[15] The flasks were mechanically shaken for 45 min; at the end of this period, another 1-ml sample was withdrawn, the cells were removed from the flask, blotted, and transferred to another flask containing 4 ml of the same incubating medium as the first. A 1-ml sample was withdrawn from this flask and then the flasks were shaken for another 45-min period. Meanwhile, the ACh content in each of the samples was being determined. At the end of the second 45-min period, another 1-ml sample was taken from each flask, the cells were transferred to new flasks, and the process was repeated for a total of four 45-min incubation periods. The pH of the incubating medium was usually measured at the end of the 45-min incubation periods; it was never lower than 6·9. The wet and dry weights of the cells were determined at the end of the earlier experiments, but since it was found, as was also found earlier by Rosenberg and Dettbarn,[16] that there was very little correlation between weight and hydrolytic activity, this was discontinued. There was good correlation between the number of cells used and hydrolytic activity. The dimensions of the cells were measured to the nearest 0·5 mm, but again there was very poor correlation between the area of the innervated membrane and the hydrolytic activity. Perhaps this indicates that each cell has approximately the same number of synapses regardless of size.

The rate of hydrolysis was calculated from the difference in the ACh concentration at the beginning and end of each 45-min period. The rates were fairly constant for the last three periods, but were usually slower for the first period, perhaps because of diffusion barriers. In any case, the first period was ignored and the rates were taken as the average of the last three periods.

The velocity of ACh hydrolysis in the control divided by the velocity of hydrolysis with an inhibitor run simultaneously was used as v/v' in the Augustinsson equation. For each of the three or four concentrations of each inhibitor used, a v/v' was obtained from four or more different experiments. These results were averaged and the standard errors were determined. Inhibitor concentrations were selected to give one or two average v/v' values below two (50 per cent inhibition) and one or two v/v' values above two. The Augustinsson method[17] was used partly because AChE exhibits substrate inhibition. The Lineweaver–Burk plot,[18] therefore, does not give a straight line,

except in the region of low substrate concentrations where the rate of hydrolysis is too slow to be reliably measured with these techniques. Also, since the Augustinsson technique requires the use of only one substrate concentration, it is possible to choose an ACh concentration where most of the hydrolysis will be catalyzed by specific (cellular) AChE rather than by connective tissue esterase.[16] The Augustinsson equation is:

$$v/v' = 1 + [I]\frac{K_S}{K_I([S] + K_S)} \quad (1)$$

where v = the velocity of hydrolysis without an inhibitor and v' = the velocity in the presence of inhibitor at a concentration of $[I]$. K_S is the dissociation constant for the substrate–enzyme complex and K_I is the dissociation constant for the inhibitor–enzyme complex. $[S]$ is the concentration of substrate. The Augustinsson equation may be derived by dividing the Michaelis–Menten equation for the velocity of an enzymatic reaction[19] by the equation for the velocity in the presence of a competitive inhibitor. It can be seen from this equation that, if $[S]$ is held constant, a plot of v/v' versus $[I]$ should give a straight line with an intercept of 1 on the v/v' axis. The slope of this line will be:

$$\frac{K_S}{K_I([S] + K_S)}$$

Therefore: $K_I = \dfrac{K_S}{m([S] + K_S)}$ where m is the slope of the line.

RESULTS

In 26 experiments (52 cells), done with an ACh concentration of 10^{-3}M and no inhibitor present, the average rate of hydrolysis was 0·68 μmole/hr/cell, with a standard deviation of 0·39 μmole/hr/cell. The variation was much greater from eel to eel than from cell to cell in the same eel, thus variation in the v/v' values was reduced by calculating them each day by using the v obtained on that day.

Figures 2–4 show the Augustinsson plots obtained for the inhibitors that were used. Figure 2 shows that a straight line with an intercept of 1 on the v/v' axis fits the data obtained using curare. This line is significant to less than the 5 per cent level, according to the analysis of variance of linear regression. Therefore, curare would appear to be a competitive inhibitor. The other four compounds appear to give curved lines, indicating that something more than simple competitive inhibition is involved. For the purposes of comparison, straight lines were drawn by the method of least squares regression by using the three experimentally determined points and the point $v/v' = 1$ at $[I] = 0$. The larger standard errors seen with the higher inhibitor concentrations reflect the fact that at high v/v' ratios the v' values are so low as to approach the limit of sensitivity of the method. The apparent dissociation constants were calculated from the slope of regression lines, as was done in the earlier work with purified enzyme.[11] The equation used is given in the materials and methods section above.

The results shown in the figures were obtained by using an $[S]$ of 10^{-3}M. This concentration of 10^{-3}M ACh was found by Rosenberg and Dettbarn[16] to be within the middle of the range where most of the hydrolysis of ACh was catalyzed by specific AChE rather than by the connective tissue cholinesterase. It is also of interest that

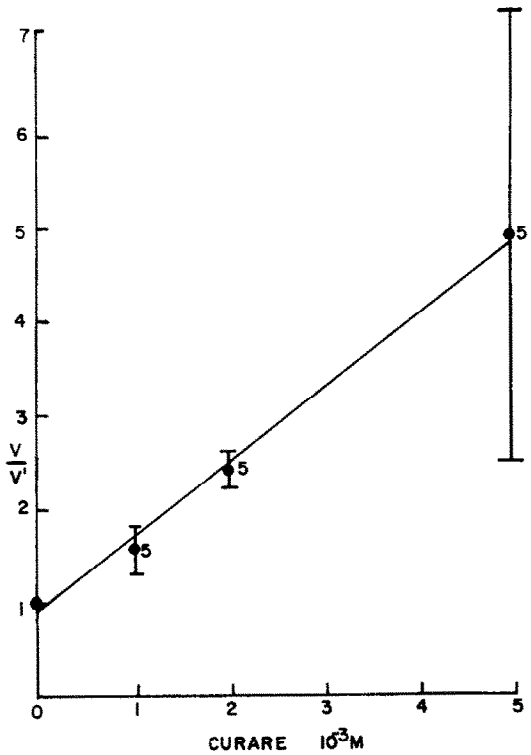


FIG. 2. The Augustinsson plot of the data obtained by using curare as an inhibitor of the AChE of intact electroplax cells. The abscissa is the concentration of inhibitor in units of 10^{-3}M. The ordinate is the velocity of hydrolysis of ACh without inhibitor divided by the velocity with inhibitor. The ACh concentration was 10^{-3}M. The points represent the mean of the number of experiments indicated by each point. The vertical bars indicate the range of the mean $\pm$ the standard error. The standard error is $\sqrt{\Sigma(x_1 - \bar{x})^2/n(n-1)}$. The straight line was drawn by the method of least squares regression.

Crawford[20] has calculated that in the region of the receptor site the ACh concentration normally reaches 10^{-3}M during the physiologically produced end-plate potential. A high concentration of ACh (8×10^{-3}M) was also tried in an attempt to study inhibition of the connective tissue cholinesterase (the AChE exhibits substrate inhibition, the connective tissue esterase does not). However, the results were so variable that they will not be presented here. A value of 2×10^{-3}M was used for K_S in the Augustinsson equation. This value was determined for intact electroplax by Rosenberg and Dettbarn[16] using a Lineweaver–Burk plot in the range of 5×10^{-4}M to $2{\cdot}5 \times 10^{-3}$M ACh. This is the range where the hydrolysis is primarily due to the specific AChE.

The apparent dissociation constants calculated from the slope of the least squares regression lines in Figs. 2–4 are given in column 3 of Table 1. In the table, the compounds are arranged in the order of their increasing potency as inhibitors of purified AChE. It may be seen that the order is almost the same for the potency of the compounds as inhibitors of AChE in intact cells. However, the order of potency of the compounds as inhibitors of the AChR is radically different.

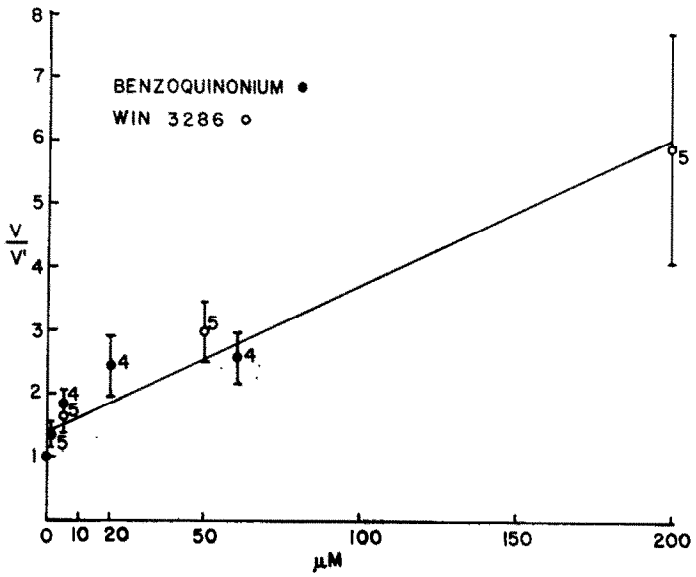


FIG. 3. The Augustinsson plot of the data obtained using WIN 3286 or benzoquinonium as inhibitors of cellular AChE. The abscissa units are μM. The rest of the legend is the same as for Fig. 2. The least squares regression lines for the two inhibitors differed by not much more than the thickness of the line, thus only one line (WIN 3286) was drawn. The slope of the two lines was slightly different, as may be seen from the calculated results in Table 1, column 3. This difference is not significant.

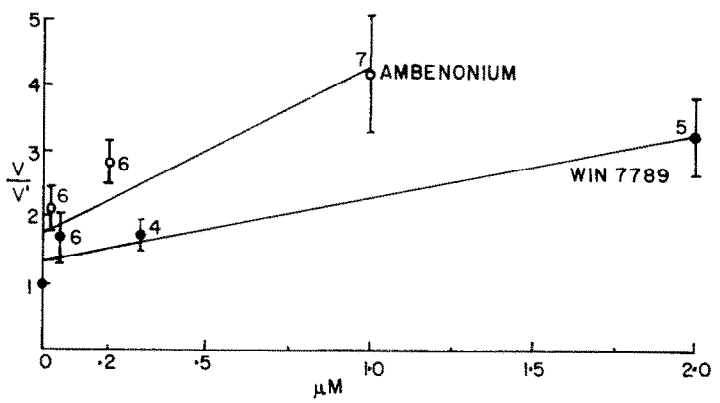


FIG. 4. The Augustinsson plot for ambenonium and WIN 7789. The abscissa units are μM. The rest of the legend is the same as for Fig. 2.

DISCUSSION

These results provide additional evidence that the receptor site for ACh is indeed different from the active site of AChE. Župančič[7] has suggested that only the anionic centers of AChE and the AChR are identical. These results would also seem to rule out that possibility, since all of the inhibitors tested are cations and thus all of them would be expected to be attracted to the anionic centers. Therefore, according to the hypothesis, they would interfere with the hydrolysis of ACh equally as well as they

interfere with the depolarization by ACh. However, they do not. As further evidence against the identity of the anionic sites, Podleski[22] has shown that the depolarizing activity of 3-hydroxyphenyltrimethylammonium is not altered by sulfonylation of the esteratic site of AChE, even though this cation is known to interact with both the anionic and esteratic sites of AChE.

In the earlier investigation,[11] the activity of these same compounds as inhibitors was studied using the receptor of the electroplax and AChE purified from the electric

TABLE 1. COMPARISON OF APPARENT DISSOCIATION CONSTANTS

Inhibitor	1 AChR (cell)	2 AChE (purified)	3 AChE (cell)	4 AChE (cell)/ AChE (purified)	5 AChE (cell)/ AChR (cell)
Curare	$2{\cdot}4 \times 10^{-7}$*	$2{\cdot}5 \times 10^{-6}$†	$8{\cdot}5 \times 10^{-4}$	340	3500
WIN 3286	$3{\cdot}4 \times 10^{-6}$‡	$2{\cdot}1 \times 10^{-7}$‡	$2{\cdot}9 \times 10^{-5}$	140	8·5
Benzo-quinonium	$1{\cdot}4 \times 10^{-8}$†	$8{\cdot}1 \times 10^{-8}$‡	$3{\cdot}0 \times 10^{-5}$	370	2100
WIN 7789	$1{\cdot}6 \times 10^{-8}$‡	$5{\cdot}0 \times 10^{-9}$‡	$7{\cdot}0 \times 10^{-7}$	140	44
Ambenonium	$1{\cdot}6 \times 10^{-6}$‡	$5{\cdot}1 \times 10^{-10}$‡	$2{\cdot}6 \times 10^{-7}$	510	0·16

* This result is taken from reference 14.
† This result is taken from reference 21.
‡ These results are taken from reference 11.

organ. The earlier study was not as conclusive as the present one because it is questionable whether it is justified to compare a purified protein with a protein that is part of a complex plasma membrane. For example, in the organized structure of the membrane, a protein might have a conformation different from that which it has in solution, due to interaction with neighboring proteins and phospholipids. This may drastically alter its reactivity. Also, intact cells are generally surrounded, to a greater or lesser degree, by permeability barriers. Nachmansohn,[23] among others, has emphasized the importance of permeability barriers in modifying the effect of small molecules on receptors in whole cells. It is quite possible that some of the compounds tested might penetrate the electroplax preparation better than others. Pennington *et al.*[24] found that when AChE is entrapped in a polymeric matrix of Silastic resin, several organophosphorus compounds become much less potent as inhibitors than when the AChE is free in solution. Rosenberg and Dettbarn[16] demonstrated that there is a significant permeability barrier even for the penetration of as small a cation as ACh to reach the AChE in the intact electroplax. It is well known that ACh and many other charged molecules do not pass through resting end-plate membranes,[25, 26] but apparently the active sites of AChE are oriented externally so that the ACh does not have to pass through the cell membrane. The permeability barrier observed by Rosenberg and Dettbarn[16] probably resides in the extracellular material covering the synapse and possibly in the circuitous route the ACh must take to diffuse through the synaptic gap.

Monod *et al.*[27] have proposed a model to account for the characteristics of allosteric proteins. This model fits the behaviour of a number of enzymes and other proteins. According to the model, the allosteric proteins have two or more stereospecific sites for one or more ligands. The binding of a ligand at one site may alter

the conformation of the protein and thus alter the dissociation constant(s) characterizing the other site(s). There is evidence that purified AChE fits this model.[6] The allosteric properties of AChE might be expected to change when the enzyme becomes part of the plasma membrane structure.

Another factor which may alter the characteristics of a membrane-bound enzyme from those of the same enzyme free in solution is the effect of neighboring fixed charges, which can drastically alter local ionic concentrations. Thus Bar-Eli and Katchalski[28] found that the *Km* may show a 20- to 50-fold increase when an enzyme is bound to an insoluble polyelectrolyte as compared to the *Km* for the same enzyme in solution.

An additional factor which may influence the accessibility of inhibitors to membrane proteins is nonspecific binding. For example, Beychok[29] has shown that curare is bound by a wide variety of proteins isolated from the electric organ of the electric eel. This nonspecific binding may lower the effective concentration of inhibitor in the region of the active site and may distort the activity vs. concentration curves.

Considering all of these factors, it is quite remarkable that the five compounds tested have almost the same order of effectiveness for inhibiting AChE both in purified solution and in the intact living cell. The fact that the Augustinsson plots give straight lines using purified enzyme[11] and curves (except for curare) using the intact electroplax is probably the result of one or more of the factors discussed above. The apparent dissociation constants are between 140 and 510 times higher for AChE in the cell than for purified AChE in solution (Table 1). Thus it appears that in both cases we are studying the same enzyme, but that in the case of the intact electroplax we are dealing with an enzyme surrounded by permeability barriers and perhaps neighboring charges. It is also possible that the conformation of the bound AChE differs from the conformation of free AChE or that the allosteric properties have changed, or both.

In contrast to the similarity between purified and cellular AChE, the cellular AChR interacts with the five inhibitors in a very different manner. The order of effectiveness is very different and the ratios of the apparent dissociation constants for cellular AChE to AChR varied from 0·16 to 3500. Another striking example of differences in the reactions of inhibitors with the AChR and the AChE of electroplax has been reported by Karlin.[30] He found that *p*-chloromercuribenzoate or dithiothreitol does not affect the *Km* or V_{max} of AChE, although they do inhibit the response of the electroplax to carbamylcholine or ACh.

Activators of the receptor also appear to have widely differing affinities or efficacies (or both) for the enzyme. For example, Bartels[31] has found that ACh and butyrylcholine are approximately equally effective activators of the electroplax AChR, even though the electroplax AChE hydrolyzes ACh many times faster than butyrylcholine. Likewise, ACh, acetylthiocholine and acetylselenocholine are hydrolyzed by electric eel AChE at similar rates, but have widely different activities for depolarizing the electroplax.[32]

All of these data seem to provide strong evidence that the active site of AChE is different from the ACh receptor site in the postsynaptic membrane of the electroplax. Presumably the same holds true for the motor end plate; as a confirmation of this, Albuquerque *et al.*[33] have recently found that a number of proteolytic enzymes can inactivate the AChE of rat or frog skeletal muscle without affecting the ACh sensitivity.

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