Biochemical Pharmacology, Vol. 23, pp. 725–733. Pergamon Press, 1974. Printed in Great Britain.

EFFECTS OF PROCAINE AND *d*-TUBOCURARINE ON THE ACTIVITY OF MEMBRANE BOUND ACETYLCHOLINESTERASE*

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(*Received* 15 *May* 1973; *accepted* 19 *July* 1973)

Abstract—The effects of *d*-tubocurarine chloride and procaine, two agents known to affect the electrobiogenesis, were tested on acetylcholinesterase activity. The compounds inhibit the catalytic activity by a more complex process than simple competitive kinetics. The results are discussed in terms of allosteric interactions.

IT HAS been suggested that acetylcholinesterase (AchE)† is an enzyme possessing in addition to the active site, peripheral non-catalytic anionic centers. Binding of Ach‡ and its analogues at these sites appears to have a regulatory effect on the enzyme activity.[1–6]

The fact that some inhibitors are non competitive or, at least, only partially competitive, has been taken as evidence that these inhibitors could bind to some site which is distinct from the active site.[7] In the case of eserine[8,9] however, the existence of an apparent non-competitive component can be accounted for by the fact that a covalent carbamyl–enzyme complex is formed. In the case of reversible inhibitors, such as trimethylammonium, tetrapropylammonium, etc. the non competitive component does not prove the existence of a 2nd site either, since the inhibitor can also bind to the acetyl–enzyme intermediate. In the case of *d*-tubocurarine and flaxedil however, more convincing evidence for binding at another site was presented by Kitz *et al.*,[12] Belleau and Di Tullio,[13] and Mooser and Sigman.[14]

In the present work, we have studied the effect of *d*-tubocurarine chloride (curare), which prevents acetylcholine from acting on the postsynaptic membrane and consequently prevents the depolarization (inhibitor of the receptor). We have also studied the effect of procaine, a local anesthetic blocking the depolarization. The results presented here show that curare exerts a slowly progressive inhibitory effect on the AchE activity. This effect may be prevented by calcium ions. Procaine exerts an immediate inhibitory effect by a complex mechanism distinct from a simple competitive inhibition. The significance of these results is discussed in terms of the known physiological effects of the above-mentioned effectors.

* This work was supported by grant No. 790 from the "Fonds de la Recherche fondamentale collective" to Professor E. Schoffeniels.

† AchE: acetylcholine hydrolase EC 3.1.1.7.

‡ Ach: acetylcholine.

METHODS

Frozen electric organs from *Torpedo marmorata* were used as the enzyme source. A piece of organ was minced with scissors during thawing and 4 vol. of 0·3 M sucrose, pH 7·0 were added to the resulting paste. The suspension was homogenized in a Potter–Elvehjem homogenizer with a loose-fitting pestle and centrifuged. The particulate fraction sedimenting between 1500 and 12,000 *g* was resuspended in 0·3 M sucrose and used as the enzyme preparation. The presence of vesicles ("microsacs") originating from fragments of electroplax membranes, was demonstrated by electron microscopy.

pH stat method. A Radiometer TTT 1 C pH stat device was used, the reaction vessel being thermostatized at 20°. The experimental conditions were the same as described by Karlin[15] except that no NaCl was present in the medium. The titrant was always 10^{-2} M NaOH and the end point was usually pH 7·0. The volume of the reaction medium was always 20 ml.

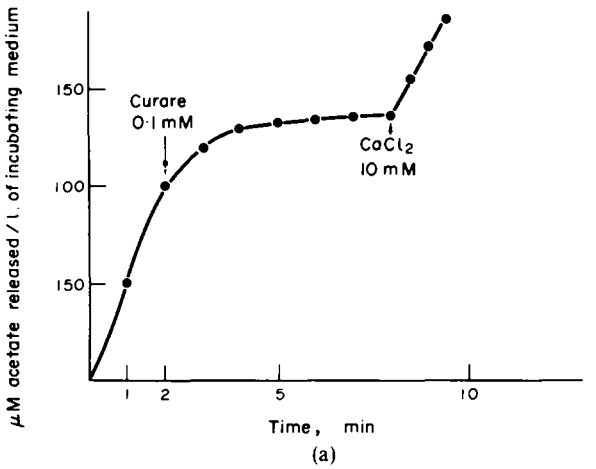
Colorimetric method. 3 ml of a reaction medium containing 1 mM ATCh,* 20 mM imidazole-maleate buffer pH 7·0, $5 \cdot 10^{-4}$ DTNB† and enough enzyme to hydrolyze about 0·2 mM ATCh/min was put in a 1 cm light path spectrophotometer cell. DTNB reacted with the thiocholine released to form a complex, the O.D. of which was 412 nm. It was verified that DTNB itself had no inhibitory effect on the enzyme activity. This is in agreement with the well-known fact that AchE exhibits little sensitivity to thiol reagents.[15,16]

Various inhibitors and activators were added to the medium during the course of the reaction which was followed at 412 nm.

RESULTS

Effects of curare. Figure 1 shows the time-course of the enzymatic hydrolysis of 1 mM Ach using the pH stat method. It can be seen that the addition of 0·1 mM curare induced a progressive decrease in the rate of hydrolysis and 3–4 min were needed before a new steady state was reached. The latter corresponds to 7 per cent of the initial rate. It can also be observed that Ca^{2+} ions greatly stimulated the enzymatic activity and allowed recovery of the activity, as previously demonstrated.[17] In this experiment we used the pH stat method which measures the rate of hydrolysis of Ach by neutralization of the equivalents of acetic acid liberated. Therefore no buffer was added. It has been shown that the presence or absence of a buffer affects kinetics data[18,19] obtained with membrane-bound AchE. This fact is mainly explained by a different binding of the protons to the buffer and to the enzyme, possibly to the active site. It was thus of interest to know if the presence of a buffer affected the inhibitory effect of curare. Experiments were performed with 1 mM ATch, in the presence of a buffer, by the colorimetric method, the activity being given by the amount of thiocholine released. The rate of ATch hydrolysis was linear during several minutes if no effector was added. The progressive inhibitory effect of curare occurred as before. Here too, Ca^{2+} 30 mM induced a marked reactivation. No discrepancy between the results obtained by the two methods was thus observed and we may conclude from this that the presence or the absence of a buffer does not affect the time course of binding of curare to AchE.

* ATch: acetylthiocholine.
† DTNB: 5·5′ dithiobis–(2 nitrobenzoic acid) Tris salt.



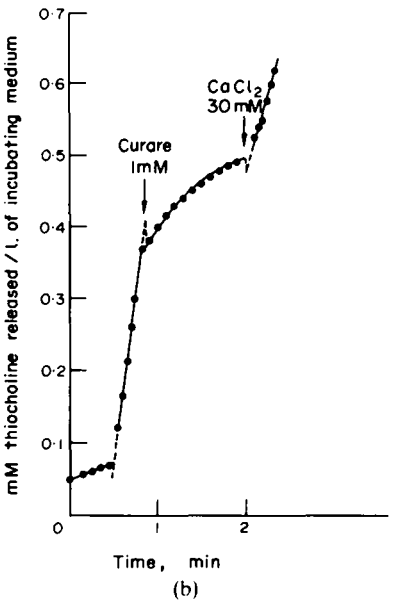


Fig. 1. (a) Time recording of the amount of 10^{-2} M NaOH added to the medium during enzymatic hydrolysis of 1 mM Ach. The curves are redrawn directly from the recordings given by the pH stat. The temperature was 20°, the pH 7·0. *d*-Tubocurarine chloride (curare) and $CaCl_2$ were added at the times indicated by the arrows. The enzyme preparation was added at zero time. (b). Effects of curare and $CaCl_2$ on the enzymatic hydrolysis of ATch at 20° estimated by absorbancy measurements at 410 nm. The incubation medium contained 1 mM ATch, 20 mM imidazole-maleate buffer pH 7·0 and $5{\cdot}10^{-4}$ M DTNB. The enzyme preparation (E) and the other reagents were added at the times indicated by the arrows. The small drop in optical density observed on addition of the reagents is due to the dilution of the medium.

However the protons play a great role in this fixation.

Indeed Fig. 2 shows that the pH greatly affects the time necessary before a new steady-state is reached after addition of curare. The results are expressed as relative rates of reaction at pH 5 and 8. It can be seen that an increase in the time required

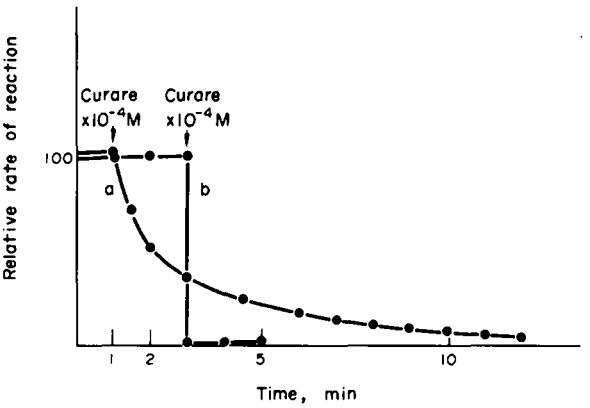


FIG. 2. Plot of the relative rate of reaction as a function of time i.e. the ratio ($100 \times V/V_0$) where V is the time derivative of the amount of 10^{-2} M NaOH added (i.e. the rate of reaction) and V_0 the initial rate of hydrolysis of 1 mM Ach by the enzymatic preparation. The temperature was 20° and pH 5 (*a* curve) or pH 8 (*b* curve).

for inhibition was observed at an acid pH. At pH 8, an immediate inhibition occurred. If we express the results of Fig. 1 (pH 7) in terms of relative rate of reaction (ordinate), the time required before a new steady-state is reached, after addition of curare, lies between that observed at pH 5 and that observed at pH 8.

Some results suggest an allosteric effect of curare.[13,20,25] It was thus interesting to consider the effect of several concentrations of curare on the rate of hydrolysis of 1 mM Ach. Figure 3 shows the inhibitory effects of increasing amounts of curare on the enzymatic hydrolysis of Ach 10^{-3} M. One possibility is that there is a cooperative binding of several molecules of curare, to the enzyme. When applied to this kinetic of inhibition by curare, the Hill equation becomes $\log (v/V_0 - v) = n_I \log I - \log K'$ where v is the velocity and V_0 the velocity without inhibitor.[21,24] The plot of $\log (v/v_0 - v)$ vs $\log I$ of the first portion of the curve gives a straight line with a slope equal to 1·33. This suggests a cooperativity and implies that the number of

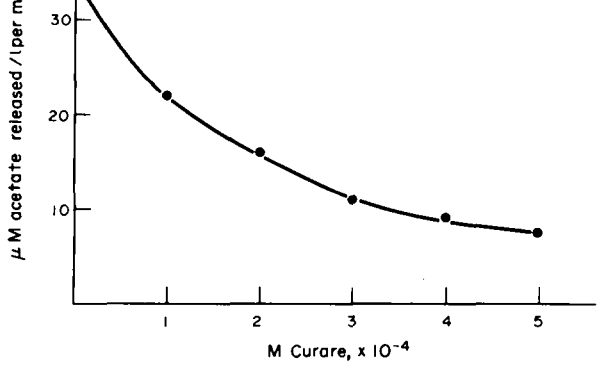


FIG. 3. Effect of various *d*-tubocurarine chloride concentrations on the AchE activity in the presence of 1 mM Ach. The temperature was 20°, pH 7.

interacting sites binding curare is greater than one, and that this phenomenon of inhibition may be due to an allosteric mechanism.

On the other hand, it is well known that a hyperbola should normally be obtained when the rate of hydrolysis of Ach is plotted as a function of substrate concentration.[2 7] In the presence of curare $2{\cdot}5\ 10^{-5}$ M, the curve has a different shape (Fig. 4). The Hill equation may be applied to this curve ($\log(V/V_{max} - V) = -\log K + n\log(S)$). If we plot $\log(V/V_{max} - V)$ vs $\log(S)$ we obtain a straight line with a slope equal to 1·3. This may indicate that in the presence of curare at least two Ach molecules bind to the enzyme protein the binding being cooperative.

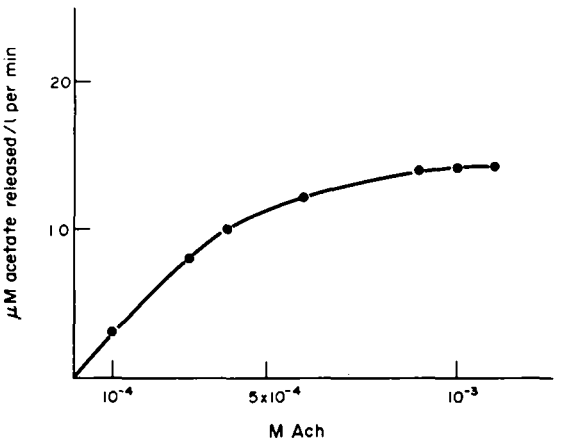


FIG. 4. Effect of Ach concentration on the AchE activity in the presence of curare $2{\cdot}5 \times 10^{-5}$ M. The temperature was 20° and pH 7·0.

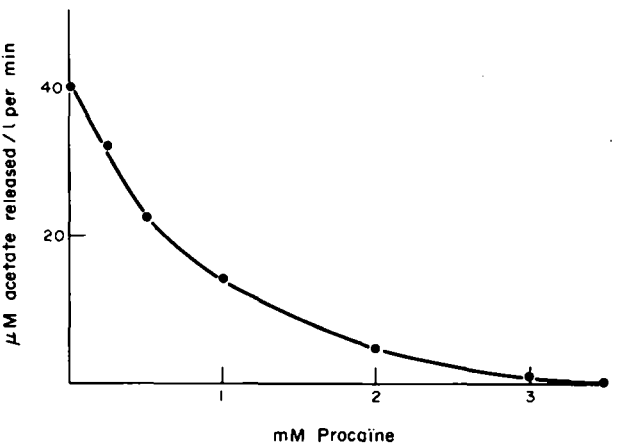


FIG. 5. Effect of various procaine concentrations on the AchE activity in the presence of 1 mM Ach. The temperature was 20°, and the pH 7·0.

Effects of procaine. With procaine, an immediate inhibitory effect was observed. Figure 5 shows the effects of various procaine concentrations on the reaction rate of hydrolysis of Ach. Procaine could inhibit the AchE activity by binding to the active catalytic site or to some allosteric sites.

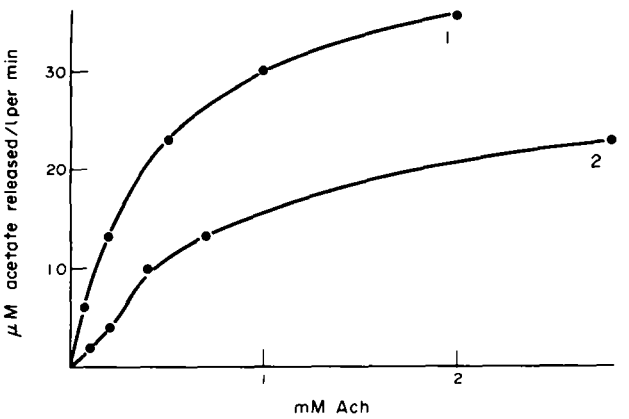


FIG. 6. Effect of Ach concentration on the AchE activity. The temperature was 20° and pH 7·0. Curve 1: control; curve 2: 1 mM procaine was added.

To test the hypothesis that procaine would be a competitive inhibitor, we have plotted the rate of hydrolysis of Ach as a function of Ach concentration in the presence and absence of procaine (Fig. 6). It can be seen that in the absence of procaine (curve 1) the curve obtained is hyperbolic. In the presence of procaine 10^{-3} M, the curve obtained differs from the classical hyperbola obtained with nonallosteric enzymes. A sigmoid shape is obtained at least at low substrate concentrations. At higher Ach concentrations, the activity increases steadily with no marked tendency to saturation. It is generally considered that the sigmoid shape of the curve giving the activity vs (S) is indicative of cooperative effects between several substrate binding sites.[21–23] It is generally assumed as the simplest hypothesis that there are n binding sites and that the concentrations of enzyme molecules containing fewer than n molecules of substrates is small.[21,44] Calculations were thus based on the equations

$$K_M = \frac{(E)(S)^n}{(ES_n)} \qquad v = \frac{V_M (S)^n}{K_M + (S)^n} \qquad \text{(Ref. 20).}$$

The empirical Hill plot of $\log (V/V_M - V)$ vs $\log S$ was used with the results shown in Fig. 6. Since the curve obtained in the presence of procaine (Fig. 6, curve 2) showed no tendency to saturation at the concentrations of Ach used, there was considerable uncertainty concerning the value of V_M. The slope of the plot was always higher than 1 whatever the value of V_M chosen in Fig. 7, being at least 1·4. This value was assumed as n for the category of sites binding Ach, suggesting that in the presence of procaine at least 2 Ach molecules must interact to explain the observed behaviour.

Simple competitive kinetics are unable to explain these results. The Hill equation, when modified, can be applied to the kinetics of inhibition by procaine.[24] In Fig. 8 we have plotted $\log (V/V_0 - V)$ vs $\log (I)$ where V_0 is the velocity without inhibitor and V the velocity of the reaction. The slope of this plot is 1·7, suggesting the binding of procaine to at least two binding sites of AchE.

DISCUSSION

As pointed out by Changeux *et al.*[2,3] there are striking similarities between the active or regulatory sites of AchE and the Ach receptor site (s) involved in electrical

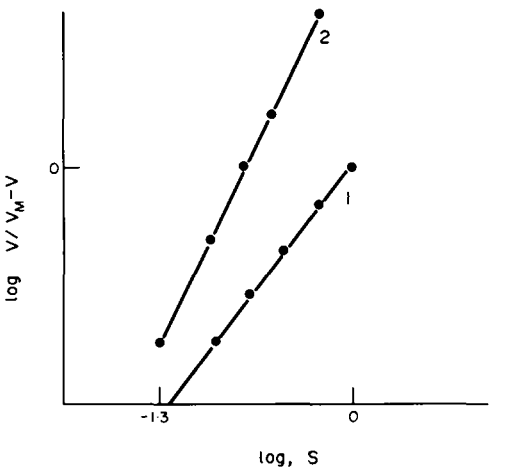


FIG. 7. Hill plots corresponding to curve 2 of Fig. 6 (plot 1) assuming that V_M = 33 μM acetate liberated per l. of incubating medium and per min (n_H = 1·4) or (plot 2) that V_M = 26 μM acetate l^{-1} min^{-1} (n_H = 2).

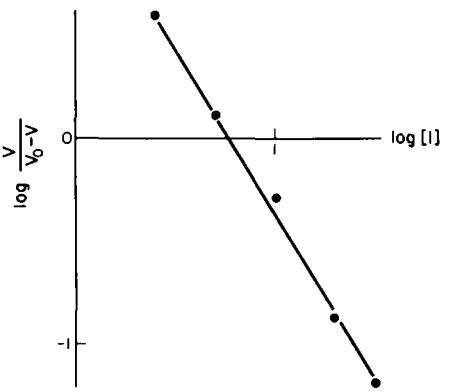


FIG. 8. Plot of log ($V/V_0 - V$) vs log (I) where V is the rate of reaction in μM acetate liberated per l. of incubation medium and per min, V_0 the initial rate in the absence of an inhibitor and (I) the concentrations of procaine in mM/l. The slope equals 1·7.

activity of the postsynaptic membranes. It is possible that regulatory sites of AchE play a role in the electrical activity of the postsynaptic membranes. We have studied the properties of non-purified but membrane-bound AchE in the presence of compounds known to affect the electrical activity, blocking the depolarization.

1. Our results show that curare induces a slowly progressive inhibitory effect on the enzymatic hydrolysis of Ach. Progressive inhibitions of AchE activity were described previously by Wilson (carbamates, sulfonates, organophosphates) and by Changeux (TDF).[2, 26–31] A covalent bond was formed between the inhibitor and the esteratic point of the catalytic site of AchE explaining the progressive nature of the inhibition. This explanation is not applicable for the inhibition observed with curare because it does not possess an electrophilic group which could form a covalent bond with the nucleophile of the esteratic site.[6,18,19,32]

The progressive inhibitory effect may be interpreted as indicating a slow change in the configuration of the enzyme induced by the fixation of the substrate or the inhibitor on peripheral sites or allosteric sites of the enzyme. Another interpretation would be that the enzyme molecules binding curare would immediately change their conformation. This change would not directly affect the enzyme catalytic activity. However in their new configuration, the enzyme molecules would tend to form complex aggregates and most of the catalytic active sites would become progressively inaccessible to the substrate. This phenomenon, well described *in vitro* with enzymes dissolved at low ionic strength and pH, should not be taken as an explanation of our data obtained with a membrane bound enzyme. Indeed the conditions *in situ* must be quite different from those *in vitro*.[43] Possible participation of a reversible aggregating AchE system as part of the macromolecular structure of electrogenetic membranes regulating ionic permeability could be suggested.[33-37]

2. Ca^{2-} ions induce an immediate and important recovery of the activity. The effects of Ca^{2-} ions have been previously described.[17,38] This reactivation observed with Ca^{2+} ions is of great interest and could have a physiological meaning. It is well known that calcium ions play an important role in bioelectro-genesis. Low amounts of calcium chloride seem to antagonize more easily the inhibitory action of the receptor inhibitors than that of the activators and Ca ions reactivate more easily AchE inhibited by curare than by carbamylcholine.[39,40]

3. The time required before a new steady-state is reached after addition of curare is greatly affected by the amounts of protons in the medium. If we suggest a change in the configuration of the enzyme to explain the progressiveness of the inhibition, it can be expected that this phenomenon should be greatly affected by pH.

4. The kinetic studies of inhibition by curare (Fig. 3) and of the activity of the enzyme in the presence of a constant amount of curare with several concentrations of substrates (Fig. 4) suggest that the phenomenon is complex and cannot be satisfactorily and quantitatively interpreted on the basis of simple competitive kinetics. The problem is complicated further by the fact that peripheral non-catalytic sites could bind Ach and most probably curare. Indeed binding of curare to allosteric sites must be postulated in order to account for the fact that the slope of the Hill plots is different from 1. This suggests some cooperativity. Cooperativity is generally accounted for by allosteric effects involving several subunits. Acetylcholinesterase is known to have a subunit structure.[41-43]

5. Our results show that procaine exerts an immediate inhibitory effect and that the curve giving the rate of hydrolysis vs several concentrations of the substrate in the presence of a constant amount of procaine has a sigmoid shape (Fig. 6). This suggests that the inhibitory effect of procaine involves a more complex process than simple competitive kinetics. Indeed binding of procaine to allosteric sites might be postulated in order to account for the appearance of a sigmoid shape.

6. The value of n calculated from the Hill equation applied to our results is at least 1·4 (Fig. 7) suggesting that at least two interacting sites bind Ach in the presence of procaine.

7. The slope of the straight line obtained from the plot of log $(V/V_0 - V)$ vs log I is 1·7 (Fig. 8) suggesting that inhibition by procaine occurs by binding of procaine to at least two sites of AchE. These results suggest some allosteric properties of AchE.

From the above results we can assume that the catalytic activity of AchE is related

to the binding of the substrate and effectors at peripheral non-catalytic sites that control the catalytic properties of the AchE. This control of an enzyme playing an important specific role at the synapse by some compounds known to affect the electrical activity at the synapse (curare, procaine, calcium ions) might have a physiological meaning. AchE and acetylcholine receptor could be closely associated and subject to mutual interactions that could well explain the properties of the synaptic function. It has been suggested that the effects of curare and procaine on the catalytic properties of AchE could be interpreted in terms of allosteric interactions.

Acknowledgements We wish to thank Professor E. Schoffeniels and Dr. P. Wins, for their helpful guidance and criticism, and Professor H. Firket for performing the electron microscope analyses.

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