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CHEMICAL DISTINCTIONS BETWEEN ACETYLCHOLINESTERASE AND THE ACETYLCHOLINE RECEPTOR

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SUMMARY

Preincubation with either *p*-chloromercuribenzoate (PCMB) or dithiothreitol was found to have no significant effect on the K_m and v_{max} of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) under conditions similar to those which resulted in a strong inhibition of the response of the intact electroplax to acetylcholine and its congeners. It is argued that these results imply that the enzyme and the receptor for acetylcholine are separate proteins.

INTRODUCTION

It has been reasonably assumed that the catalytic site of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) and the binding site of the physiological receptor for acetylcholine have properties in common[1], and on the basis of this assumption acetylcholinesterase has been used as a model for the receptor (*e.g.*, ref. 2). Several workers have gone beyond this general idea, however, and have suggested that the enzyme and the receptor are one and the same protein and that the two active sites are either fully or partially coincident[3–7]. This hypothesis, although made plausible by the localization of the enzyme in the right places and some similarities in the specificities of the two sites, cannot easily be reconciled with the sometimes large quantitative differences in the apparent affinities of the two sites for various receptor activators and inhibitors. For example, the apparent dissociation constant of the receptor–acetylcholine complex in the isolated electroplax of *Electrophorus electricus* is approx. $5 \cdot 10^{-6}$ M (ref. 8), whereas the K_m of acetylcholinesterase from the same cell is approx. $1 \cdot 10^{-4}$ M. For bis-quaternary ammonium inhibitors as well, the apparent affinities of the two sites differ, and, in some cases, minor modifications of these inhibitors shift the two affinities in opposite directions[9]. Furthermore, irreversible inhibitors of the enzyme potentiate the effect of acetylcholine and do not block the effects of non-hydrolyzable congeners of acetylcholine, which for steric reasons makes it unlikely

Abbreviation: PCMB, *p*-chloromercuribenzoate.

that the two sites could be fully coincident. Finally, it has been shown that sulfur and selenium analogs of acetylcholine behave differently towards enzyme and receptor[10], again implying that the two sites could not be, at least, fully coincident. NACHMANSOHN[11] has pointed out that these lines of evidence favor the existence of two separate sites (but not necessarily two separate proteins). Since there is uncertainty about the meaning of apparent dissociation constants derived from dose–response curves, no conclusive argument may be based solely on such data.

Evidence of a somewhat different nature (although still based partially on dose–response curves) is presented here. The argument on which the interpretation of this evidence is based is that if the active sites of the enzyme and the receptor were on the same protein molecule, and especially if they were overlapping, a chemical reaction which perturbed one site should also perturb the other. It has recently been shown that *p*-chloromercuribenzoate (PCMB), reacting with sulfhydryl groups, and dithiothreitol, reacting with disulfide bonds, can each, when added briefly and at low concentrations to the innervated side of the intact electroplax, inhibit the depolarization induced by acetylcholine, carbamylcholine, or trimethylbutylammonium ion[12]. The effect appears as a 3–4-fold increase in the apparent dissociation constant of the activator–receptor complex. The inhibition can be reversed by specific chemical reactions but not by washing. A likely interpretation of these results is that the receptor is a protein containing reactive sulfhydryl and disulfide groups and that reactions with these groups perturb the binding site, decreasing its affinity for acetylcholine and its congeners. The effects of PCMB and of dithiothreitol on the K_m and v_{max} of acetylcholinesterase was therefore determined.

The enzyme was tested in two forms, both prepared from the electric tissue of *E. electricus*. One form was soluble. The other, prepared by subcellular fractionation, was insoluble and membrane-bound[13]. It seems reasonable to assume that if complex interactions between different sites on the same or different molecules exist *in vivo*, such interactions would more likely be preserved in large membrane fragments than in solution. The results show a lack of an effect of the receptor-perturbing reagents on acetylcholinesterase and a near identity of the K_m of the enzyme in the two forms.

METHODS

The acetylcholinesterase solution, prepared by Dr. W. LEUZINGER, had a specific activity of 30 mmoles acetylcholine hydrolyzed per h per mg protein. (The fully purified enzyme has a specific activity in the same units of 750 (ref. 14).) The insoluble acetylcholinesterase was present in subcellular Fraction 4A (ref. 13) and had a specific activity (determined as in ref. 13) of approx. 7 mmoles acetylcholine hydrolyzed per h per mg protein. This fraction was stored in small aliquots at −20° and was thawed once, just prior to its use.

Aliquots of either enzyme preparation were preincubated with PCMB or dithiothreitol as indicated in Tables I and II. At the end of the preincubation, the preincubation mixture (vol. 1 ml) was diluted with 100 ml of 0.15 M NaCl containing 0.01% gelatin, and added to a jacketed reaction vessel of a Radiometer titrator. The titrator was operated as a pH-stat. The end point was pH 7.00–7.02. The titrant was 0.01 M NaOH. The temperature was 30°. The solution was stirred by a glass propellor stirrer. N_2 was passed continuously over the solution. The pH of the solution was brought to

TABLE I

EFFECT OF PCMB AND OF DITHIOTHREITOL ON MEMBRANE-BOUND ACETYLCHOLINESTERASE

The variable composition of the preincubation mixtures and the times of incubation are given below. In addition, all mixtures contained 0.1 or 0.2 ml of Fraction 4A and 0.008% gelatin. Controls had the same composition as the test mixtures except for the absence of PCMB or of dithiothreitol. The total vol. was 1 ml. The temperature was 30°. The preincubation was ended by dilution with 100 ml of 0.15 M NaCl *plus* 0.01% gelatin, in which K_m and v_{max} were determined. Results are expressed as the mean ± S.E.M.

Preincubation	*Number of Expts.*	K_m *(μM)*	v_{max} *(μmoles hydrolyzed per min per mg protein)*
0.5 mM PCMB, 120 mM NaCl, 6 mM potassium phosphate (pH 7.4); 5 min	4	86 ± 3	46 ± 4
Control	3	84 ± 4	45 ± 4
0.5 mM PCMB, 120 mM NaCl, 2 mM Tris (pH 8.0); 5 min	3	75 ± 3	48 ± 1
Control	3	76 ± 3	45 ± 4
1 mM PCMB, 135 mM NaCl, 8 mM potassiumphosphate (pH 7.6); 10 min	7	96 ± 2	52 ± 5
Control	7	97 ± 2	48 ± 4
1 mM dithiothreitol, 120 mM NaCl, 4 mM Tris (pH 8.0); 10 min	13	80 ± 1	51 ± 2
Control	9	80 ± 2	47 ± 2
2 mM dithiothreitol, 135 mM NaCl, 8 mM Tris (pH 8.0); 20 min	6	74 ± 2	48 ± 4
Control	5	82 ± 4	40 ± 7

TABLE II

EFFECT OF PCMB AND OF DITHIOTHREITOL ON SOLUBLE ACETYLCHOLINESTERASE

The preincubation mixtures contained, in addition to the substituents listed below, 5 μl of the acetylcholinesterase preparation containing 0.2 mg protein/ml, 150 mM NaCl, and 0.01% gelatin. The procedure is as in Table I.

Preincubation	*Number of Expts.*	K_m *(μM)*	v_{max} *(μmoles hydrolyzed per min per ml enzyme)*
1 mM PCMB, 5 mM potassium phosphate (pH 7.6); 10 min	3	81 ± 1	102 ± 8
Control	3	85 ± 3	104 ± 2
1 mM PCMB, 5 mM Tris (pH 8.0); 10 min	3	73 ± 3	107 ± 5
Control	2	72 ± 1	98 ± 5
1 mM dithiothreitol, 10 mM Tris (pH 8.0); 10 min	8	78 ± 3	98 ± 3
Control	7	82 ± 2	95 ± 4

7.0 and approx. 5 min after the dilution of the preincubation mixture the first aliquot of substrate (0.1 M acetylcholine bromide) was added. Five aliquots of substrate were added at approx. 2-min intervals[15]: 0.05, 0.05, 0.1, 0.2 and 0.6 ml, giving approx. 0.5, 1, 2, 4, and $10 \cdot 10^{-4}$ M acetylcholine in the reaction vessel. At the lowest substrate concentration approx. 7% of the total substrate was hydrolyzed, and at the highest substrate concentration, approx. 1%. No corrections were made for product inhibition. The rate of hydrolysis was taken as the average slope of the line of the Titrigraph (Radiometer, SBR2C), recording the addition of NaOH as a function of time. The rate of hydrolysis in the absence of enzyme, at all concentrations of substrate used, was negligible.

The data was plotted according to the form of the Michaelis–Menten equation[16]:

$$v = v_{max} - K_m \left(\frac{v}{S}\right)$$

The substrate concentration during an interval was taken as the sum of the initial and the final concentrations divided by 2. A straight line was drawn through the points by eye, and v_{max} and K_m determined by the intercept on the 'v' axis and by the slope, respectively.

RESULTS AND DISCUSSION

The results summarized in Tables I and II show that there is no significant effect of pretreatment with PCMB or with dithiothreitol on the K_m or v_{max} of acetylcholinesterase, either in solution or bound to membrane. Either 0.5 mM PCMB in a Ringer's solution (pH 7.8) applied for 5 min or 1 mM dithiothreitol in a Ringer's solution (pH 8.0) applied for 10 min to the intact electroplax increased the apparent dissociation constant of the receptor–carbamylcholine complex at least 3-fold[12]. Clearly, the effect of these reagents on acetylcholinesterase is negligible in comparison, even at twice the concentration and twice the time of exposure. If the effects of PCMB and of dithiothreitol observed in the intact electroplax are actually due to reactions with the receptor, then the lack of an effect on acetylcholinesterase presents great difficulties for the hypothesis that the active sites of the receptor and the enzyme are even partially coincident. These results also make it seem unlikely, although it remains conceivable, that the two sites could be completely separate but still on one protein molecule. Another interesting aspect of the data is that membrane-bound acetylcholinesterase behaves kinetically, at least at an ionic strength of 0.15, as if it were in solution: the mean of all determinations of K_m for the soluble acetylcholinesterase (Table II) is 79 μM, and that for membrane-bound acetylcholinesterase (Table I), 84 μM.

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The agreement between the K_m for the soluble enzyme and that for the membrane-bound enzyme may well be fortuitous. During hydrolysis, both the pH and the substrate concentration are lower in the vicinity of the membrane-bound enzyme than in the bulk solution (Silman and Karlin, manuscript in preparation). It can be

shown that the K_m is changed in opposite directions by these two effects, and hence they tend to cancel. The conclusions with regard to the receptor are not changed by these findings.

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