KINETIC RESPONSE OF A MEMBRANE-BOUND ACETYLCHOLINESTERASE TO CHOLINERGIC ACTIVATING AND BLOCKING AGENTS

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1. Introduction

Acetylcholine (ACh*) and other cholinergic agents are supposed to induce a conformational change in the ChR protein. With the nicotinic receptor of the electroplax membrane, sigmoid dose-response curves were found indicating a cooperative interaction between ChR sites [1]. Obviously, a ChR protein should display analogous behaviour. The present approach to the question of the nature of ChR is based on the working hypothesis that anionic centres – not the esteratic ones – of ChE are identical with ChR sites [2–5]; it was repeatedly stressed that only ChE built-in into excitable membranes are liable to function as ChR, while a purified enzyme might have lost this property [5, 6]. According to this hypothesis, ChE bound to excitable membranes are expected to display an analogous kinetic behaviour in the presence of various substances, as do ChR. The aim of the present study was to test whether the assumed kinetic behaviour can be found with acetyl-ChE (EC 3.1.1.7) built-in into motor endplates of mouse diaphragms.

* *Abbreviations*:
ACh : acetylcholine;
Amb : ambenonium;
ChE : cholinesterases;
ChR : cholinoreceptor;
DPDA: *N,N'*-diisopropylphosphorodiamine;
E : eserine;
Gal : gallamine;
MCh : acetyl-β-methylcholine;
TC : *d*(+)tubocurarine;
TEA : tetraethylammonium;
TMA : tetramethylammonium.

2. Methods

Membrane-bound acetyl-ChE preparation: White mice of either sex, weighing 25–30 g, were sacrificed with ether; their diaphragms were excised and rinsed with Ringer fluid; 2.5 g of fresh tissue were sheared in 15 ml of Ringer fluid for 90 sec in a Virtis 23 blender at 0° and a speed of 30 scale units. The suspension was centrifuged at 25,000 *g*, for 30 min at 4°, the supernatant discarded and 0.3–0.6 g of the sediment suspended in 15 ml of the reaction mixture, containing DPDA (1 μM), unless otherwise stated. In control experiments, a purified acetyl-ChE preparation (Worthington Biochem. Corp.) was used. The composition of Ringer fluid was the same as described previously [3], except that the NaCl concentration was raised to 9 g/l and the pH adjusted to 7.4 with 2 mM tris-HCl. Enzyme activity was measured in 15 ml of the reaction mixture by continuous pH-stat titration (5–10 mM NaOH) at pH 7.4 and 38°. The initial concentration of the substrate was kept constant by adding it during titration. Inhibitors were added before the substrate to final concentrations which reduced the hydrolysis of ACh (0.1 mM) by 30–50%.

3. Results

The suspension consists of muscle fibre fragments, 50–300 μm long, on some of which motor endplates can be visualised after incubation with Koelle medium. The supernatant contains less than 10% of the total enzyme activity. Eserine (10 μM) completely inhibits the hydrolysis of ACh, MCh, butyrylcholine, and propionylcholine at concentrations from 0.3 to 20

mM (all measured without DPDA). After incubation with DPDA, the preparation does not hydrolyse butyrylcholine, whereas the hydrolysis of MCh is unaltered by DPDA.

In muscle fibre fragments without endplates, the ChE activity per unit weight is about 20% of that in fragments with endplates. The activity outside the endplates is completely inhibited after 30 min of incubation with DPDA. With ACh as substrate, our enzyme preparation display an optical activity at pS = 2.8 and obeys Michaelis–Menten kinetics at suboptimal substrate concentrations down to pS 5.1.

In the presence of TC (1 μM), gallamine (2.5 μM), or TEA (1 mM), the hydrolysis of ACh no longer obeys Michaelis–Menten kinetics. If MCh is used as substrate, the same deviation is found in the absence of inhibitors. Eserine (0.1 μM), TMA (3 mM) or ambenonium (2 nM) do not alter the usual kinetics. Results plotted according to the Hill equation, yield Hill coefficients (n_H) of either ∿1 (0.9–1.1) or ∿2 (1.8–2.1). The main results are summarised in table 1 and some are illustrated in fig. 1.

Table 1
Type of kinetics of acetylcholinesterase built-in into mouse diaphragm endplates.

Substances	Michaelis–Menten kinetics	n_H
ACh (8 μM–0.5 mM)	obeyed	∿1
MCh (63 μM–1 mM)	not obeyed	∿2
ACh + TC (1 μM)	not obeyed	∿2
ACh + Gal (2.5 μM)	not obeyed	∿2
ACh + TEA (1 mM)	not obeyed	∿2
ACh + TMA (3 mM)	obeyed	∿1
ACh + Amb (2 nM)	obeyed	∿1
ACh + E (0.1 μM)	obeyed	∿1

n_H: the Hill coefficient at substrate concentrations lower than about 0.1 mM.

4. Discussion

In preparing ChE integrated into the motor endplate membrane, most careful handling of the enzyme is necessary in order not to disturb the delicate conformation of the protein under study. Hence, drastic interference with the protein configuration, such as

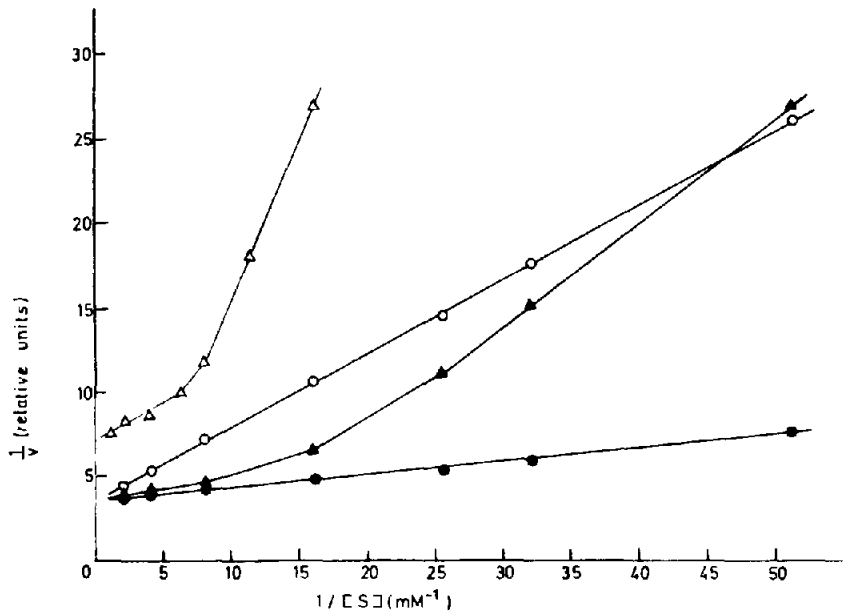

Fig. 1. Lineweaver–Burk plot for the hydrolysis of ACh and MCh by acetyl-Che bound to motor endplates. ●——●: ACh; ○——○: ACh in the presence of eserine (0.1 μM); ▲——▲: ACh in the presence of *d*(+)tubocurarine (1 μM); △——△: MCh.

freezing, is not allowed. With our preparation the whole procedure consists in shearing the diaphragms. The final inhibitor concentrations are constant as they greatly exceed those of the active centres of acetyl-ChE. The possible binding of inhibitors to other molecules in the preparation is irrelevant since the only measured parameter is the hydrolysis of ACh or MCh, catalysed by acetyl-ChE.

The described membrane-bound enzyme preparation is characterized as acetyl-ChE by the pS-activity curve and by its selectivety regarding substrates and inhibitors. The most interesting result is the change of the rectangular hyperbola in the activity–substrate concentration plot to an S-shaped curve if one of the tested antagonists of nicotinic ChR is present; the same is true without antagonists if MCh is used as substrate. This change in kinetics is best demonstrated with the aid of the Lineweaver–Burk plot (fig. 1). In control experiments with the purified acetyl-ChE preparation, the Michaelis–Menten kinetics is obeyed both with ACh and MCh regardless of the presence of ChE inhibitors or cholinergic blocking agents.

A hyperbolic curve indicates that the affinity of individual binding sites for the ligand is independent of whether the neighbouring sites are occupied by the ligand or not; an S-shaped curve, on the other hand, points to a conformation where the binding of one ligand molecule to one of the subunits influences the affinity of the still unoccupied subunits for the ligand. With ChR activators and blocking agents, hyperbolic and S-shaped dose-response curves have been found and interpreted in the above way [1]; Our results, likewise, allow a similar interpretation: with ACh as substrate the membrane-bound acetyl-ChE displays a structure with independent binding sites as indicated by the Hill coefficient $\sim$1.0. The transition of the enzyme to the structure with independent sites is prevented by TC (1 μM), gallamine (2.5 μM), or TEA (1 mM); this effect is found with concentrations at which the substances are effective as antagonists of nicotinic ChR and which are not related to their anti-ChE potency. In all three instances the Hill coefficient is close to 2, pointing to two allosterically coupled subunits; this result is in agreement with the suggestion that the acetyl-ChE molecule is a dimeric hybrid [7]. On the other hand, TMA (3 mM), itself a ChR activator, does not prevent the transition of the enzyme to the structure with independent sites, though the structurally very similar TEA does. Eserine (0.1 μM) which in concentrations lower than about 0.1 mM is not a ChR antagonist [8], does not oppose the transition of the membrane-bound enzyme to the structure with independent sites; likewise, ambenonium (2nM) which is not an antagonist of nicotinic receptors at the concentration used [9], does not prevent the transition. The analogous kinetic behaviour of nicotinic ChR and acetyl-ChE bound to muscle endplates can be further shown by using MCh, a weak activator of nicotinic ChR, as substrate; this ester does not induce the transition of the enzyme from the structure with interdependent to independent sites.

Except for eserine, the cationic heads of the substances tested are bound to the anionic centres of acetyl-ChE; all eight substances, including eserine, induce, prevent or allow kinetic behaviour in the described acetyl-ChE, analogous to that induced, prevented or allowed by them in the nicotinic ChR. Thus, the above results support the working hypothesis that anionic centres of ChE, built into excitable membranes, are identical with ChR sites on the same macromolecule.

It is conceivable that the conformational change in the quaternary structure of the receptive enzyme suggested above is but one case of a wider principle of operation of receptors. The next obvious step is to investigate a tissue with muscarinic response to ACh; such a study is under way.

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