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A NEW APPROACH TO THE PROBLEM OF THE NATURE OF CHOLINORECEPTOR*

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INTRODUCTION

K a r a s i k (1946) was the first to attribute the cholinoreceptor (ChR) function to negative groups on the cholinesterase (ChE) molecule, but he did not further develop this idea. The present study of the nature of the ChR is based on the working hypothesis that anionic centres of ChE function as (Župančič, 1953). To avoid any possible misunderstanding, it should be emphasized that it is the anionic centres and **not** the esteratic centres which are assumed to be identical with ChR.

By 'anionic centres' are meant the negatively charged groups on the active surface of ChR plus the auxiliary subcentres which together form the receptor site for the methylated cationic heads of acetylcholine (ACh) molecules.

The binding of the quaternary head of ACh to the anionic site of ChE, which is not only located on but is a part of the subsynaptic membrane, is assumed to induce a change in the enzyme which, in turn, triggers an increased flux of ions across the excitable membrane. This change in the receptor protein was postulated to be a conformational change (Nachmansohn, 1955), a suggestion which was elaborated by Belleau (1964) and Changeux et al. (1967).

According to our hypothesis, the ChR protein which is conformationally changed under the influence of ACh, is identical with ChE built-in into the excitable membrane; the purpose of the present study was to find out whether such a conformational change of ChE is effected or not.

As has been previously pointed out, in a study of the behaviour of ChE in vivo, purified enzyme preparations must be considered to be artifacts (Župančič, 1967;) hence, only membrane-bound ChE preparations can be used for the purpose outlined above.

METHODS

White mice of either sex, weighing 25 — 30 g were sacrificed with ether; diaphragms were excised and rinsed with Ringer fluid; after

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adding 15 ml of Ringer fluid per diaphragm, they were chopped for 90 sec in a 'Virtis 23' blender at 0° and a speed of 30 scale units; subsequently the suspension was centrifuged for 30 min. at 4° and 25000 g; the supernatant was discarded and the rest suspended in 15 ml of Ringer fluid per diaphragm. After incubation for 30 min with NN' -diisopropylphosphorodiamide anhydride (DPDA, 1 μM), the suspension was used as an acetyl-ChE (AChE) preparation. The composition of the Ringer fluid was the same as previously described (Župančič, 1964), except that the NaCl concentration was raised to 9 g/l and the pH adjusted to 7,4 with Tris. Enzyme activity was measured with NaOH (10 mM) in 15 ml of reaction mixture by continuous pH-static titration, at pH 7,4 and 38°. During titration volumes of ACh (10 mM), equal to the volume of consumed NaOH, were added.

RESULTS

The suspension consists of muscle fibre fragments, 50 μ — 300 μ long, on some of which motor end-plates can be visualised after incubation with Koelle medium.

The supernatant contains less than 10% of the total enzyme actitity.

Eserine (10 μM) completely inhibits the hydrolysis of ACh, butyrylcholine, propionylcholine and acetyl-β-methylcholine at concentrations from 0,3 mM to 20 mM (all measured without DPDA).

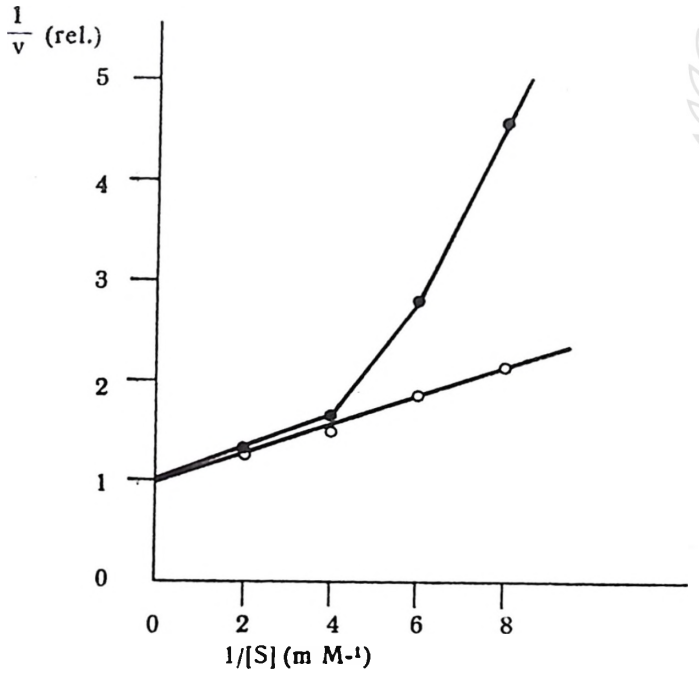

Fig. 1.
Lineweaver-Burk plot; open circles: acetylcholine; full circles: acetylcholine in presence of d-tubocurarine (2,5 μM).

In muscle fibres sections without motor end-plates the ChE activity per unit weight, is 20 — 25 % of that in sections with end-plates. The activity outside end-plates is completely inhibited after 30 min of incubation with DPDA (μM).

With ACh as substrate, our AChE displays an optimal activity at pS = 2,8. It obeys the Michaelis-Menten kinetics (measured at suboptimal substrate concentrations from pS 3,3 to 4,5); Km = 2×10^{-4} M V max = 0,2 enzyme units per diaphragm.

In the presence of d-tubocurarine (TC) at concentrations from 10 μM to 1μM, the hydrolysis of ACh does no longer obey the Michaelis-Menten kinetics (Fig. 1). On the other hand, no such deviation is found in the presence of ambenonium producing the same degree of inhibition (2 nM).

DISCUSSION

In preparing ChE built-in into the membrane most careful handling the enzyme is called for in order not to disturb the delicate native conformation of the protein to be studied. For that reason no drastic interference with the protein configuration, such as freezing, is allowed and changes in pH, ionic strength, and ionic composition were avoided as much as possible; thus, the preparations of membrane-bound ChE such as described by McCollester (1962), Rosenthal et al. (1965) or Namba and Grob (1968) do not satisfy these conditions enough rigorously. With our preparation the whole procedure consists only in chopping and centrifuging the diaphragms.

Our enzyme preparation is characterized as AChE by the pS-activity curve, by the value of Km = 2×10^{-4} M and by selective sensitivity to inhibitors. Since in the white mouse diaphragm the number of end-plates (Waser, 1966) as well as the number of AChE active centres is known (Rogers et al., 1966) the catalytic center activity of our preparation could be determined and was found to equal $1{,}2 \times 10^{6}$: this value is higher than the one obtained by Namba and Grob (1968) with ChE of end-plate membranes of rat tibialis anterior muscle (1.6×10^{5}).

The most interesting of our findings is that in the presence of TC the rectangular hyperbola in the activity-substrate concentration plot is changed into an S-shaped curve; this change is best demonstrated with aid of the Lineweaver-Burk plot (Fig. 1). It should be noted that this effect of TC is not found with purified AChE preparations.

An S-shaped curve points to a functional unit of at least two subunits where by the binding of one ligand molecule to one subunit influence the affinity of the remaining free units to the ligand, whereas a hyperbolic curve means that the affinity of separate binding sites is independent of whether the neighbouring sites are occupied or free. The transition from the form with interdependent sites to the form with independent sites reflects the underlying conformational change in the protein.

In the interpretation of our results, ACh is assumed to induce the conformational change in AChE; as can be seen from Fig. 1, this change results in functionally monomeric subunits, which do not influence each other's affinity to ACh. On the other hand, TC prevents ACh from inducing such a conformational change; in the presence of TC, the ACh-binding

sites of AChE influence each other as functionally interdependent oligomers and it is by this stabilizing effect that TC antagonises the action of ACh. Our interpretation means that a positive feedback operates at low substrate concentrations, for the binding of ACh induces a functional deaggregation of the oligomeric subunits, as a consequence of which the affinity of now monomeric subunits to ACh is increased.

The S-shaped oxygen dissociation curve of hemoglobin found in many species has the advantage of favouring the unloading of oxygen at low pressures. With ChR the unloading of the substrate is achieved by hydrolysis, which, we assume, is catalysed by the same macromolecule (ChE). After hydrolysis of ACh, the ChR monomeric subunite must reaggregate to the oligomeric conformation, which we consider to be thermodynamically less probable and thus needs special energy yielding mechanisms. Whether the proposed aggregation and deaggragation of subunits are only functional or whether some weak bonds are formed and broken, cannot be decided and, as a matter of fact, is irrelevant for our interpretation. Reversible aggregation and deaggregation of purified AChE fractions have been described by Grafius and Millar (1965, 1967); these authors ponder on the possible functional significance of reversible aggregation--deaggregation of ChE in vivo. Gerhart and Pardie (1963) have shown that native tetrameric aspartate transcarbamylase shows an S-shaped activity-substrate concentration curve; on treatment with heat, the enzyme deaggregates to monomers obeying the Michaelis-Menten kinetics; along with the classic example of oxygen equilibrium curves of various myoglobins and haemoglobins, such results support our suggested interpretation.

The following — admittedly tentative — model on the mode of operation of ChR is proposed:

1 — The anionic centres of ChE built-in into the excitable membranes are the ChR sites of the same macromolecule.

2 — In the excitable state this macromolecule has several functionally interdependent binding sites (oligomeric conformation).

3 — ACh or other ChR activators induce a transition from the oligomeric to a functionally monomeric conformation.

4 — The functional deaggregation of subunits triggers rapid ion movements across the membrane (excitation or, possibly, also inhibition).

5 — After hydrolysis of the substrate catalysed by the esteratic sites of the same macromolecule, the monomeric conformation normally must reaggregate to the oligomeric, thermodinamically less probable, conformation.

6 — The ability of an activator to induce the conformational change referred to above, is what pharmacologists described as efficacy or intrinsic activity.

7 — TC or other cholinolytics stabilise the oligomeric conformation and thereby oppose or prevent the activator-induced transition to the monomeric conformation.

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NOV PRISTUP PROBLEMU PRIRODE HOLINORECEPTORA

KRATAK SADRŽAJ

U cilju ispitivanja prirode holinoreceptora pripremljen je preparat nativne acetilholinesteraze, ugrađene u motorne pločice diafragme miša. Ovaj enzim ima Km = 2×10^{-4} M i ravna se po Michaelis-Mentenovoj kinetici. U prisustvu d-tubokurarina, medutim, aktivnost enzima ne ravna se više prema pomenutoj kinetici. Ta činjenica ukazuje na konformacione promjene, koje izaziva acetilholin, a sprečava d-tubokurarin. Ovaj rezultat podkrepljuje hipotezu o identičnosti holinoreceptora sa anjonskim centrima holinesteraza, ugrađenih u podražljive membrane. Predložena je hipoteza o načinu djelovanja holinoreceptora.

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