SPECIFIC BINDING OF ACETYLCHOLINE TO ACETYLCHOLINESTERASE IN THE PRESENCE OF ESERINE

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1. Introduction

In the course of our pursuit of the physiological receptor for acetylcholine (ACh) we have studied, by equilibrium dialysis, the reversible binding of ACh to membrane fragments isolated from electric tissue of *Electrophorus electricus*. In this letter we show that under conditions where acetylcholinesterase (AChE) is inhibited by eserine (physostigmine) the reversible binding of ACh to a protein present in the membrane fragments can be demonstrated. This protein is identified as AChE.

2. Methods

Membrane fragments derived from the innervated face of electroplax cells were purified by sucrose gradient centrifugation after homogenisation of fresh electric tissue from *E. electricus*. Pure AChE (specific activity: 770 mmoles Ach/mg/h) was prepared from the same tissue by the method of Leuzinger and Baker [1]. Equilibrium dialysis experiments were performed at room temperature following exactly the technique used by Gilbert and Müller-Hill for the isolation of the *Lac* repressor [2]. Routinely the extracts were first dialysed against 10^{-5} eserine in 10^{-3} M Na phosphate pH 7.0 in order to inhibit AChE. The bags were then equilibrated for one to two hours with a solution of radioactive ACh in the same medium. Bound ACh was estimated by measuring the radioactivity of the bag content and of the external fluid. Proteins were assayed by the method of Folin.

3. Results and discussion

Fig. 1 and table 1 show that ACh strongly binds to membrane fragments in the presence of 10^{-5} M eserine in 10^{-3} M Na phosphate pH 7.0. A similar effect is observed when phospholine (diisopropyl-phosphoryl-thiocholine) is used instead of eserine for the inhibition of AChE. As indicated on table 1 the membrane component which accounts for ACh binding is inactivated by a 15 min exposure at 60°C and by a one hour incubation at pH 2.0 or 12.0; it is digested by pronase but resists to pancreatic lipase, trypsin and chymotrypsin. This component is thus a protein. In order to identify this protein we centrifuged membrane fragments dissolved in 1% deoxycholate on top of a sucrose gradient established in 10^{-3} M Na phosphate pH 7.0 supplemented with 1‰ deoxycholate. Both ACh binding and AChE activity were assayed in each fraction collected after centrifugation. Fig. 2 shows that the ACh binding protein sediments at exactly the same velocity as AChE.

We therefore tested the binding of ACh to pure AChE prepared from electric tissue and found, indeed, that the pure protein binds ACh in the presence of 10^{-5} M eserine. In fig. 1 the binding curves of ACh to both membrane fractions and pure AChE are compared; the two curves superimpose almost exactly.

A striking quantitative feature of ACh binding to AChE is that, in the present experimental conditions, the binding curve does not follow a simple Langmuir isotherm; there is no tendency for saturation at high ACh concentration; above 10^{-6} M free ACh the amount of bound ACh increases almost linearly with the concentrations of free ACh; the Scatchard plot of

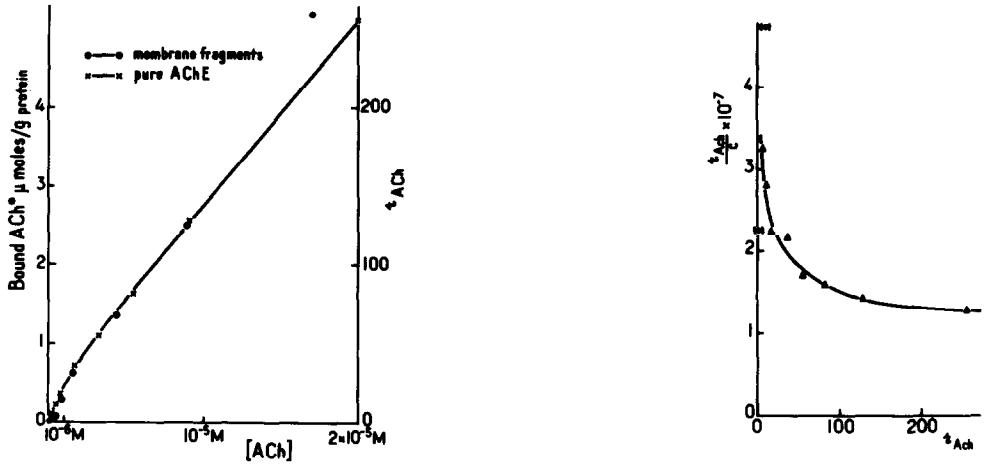

Fig. 1. Binding of ACh to membrane fragments and pure AChE both from electric tissue. Left: ACh bound per g protein is plotted as a function of free ACh concentrations. Right: Scatchard plot of the binding data relative to pure AChE; r_{ACh} is the number of ACh molecules bound per molecule of AChE (M.W. = 2.6×10^5) [6] and c is the free concentration of ACh expressed in moles per liter. AChE concentrations are estimated spectrophotometrically (ϵ = 16.1 [7]) and chemically (Folin reaction, and biuret).

Table 1
Some properties of the membrane component binding ACh in the presence of eserine.

	Bound ACh % control
10^{-6} M phospholine instead of 10^{-5} M eserine *	56.
heated at 60°C for 15 min	4.5
0.5 mg/ml pronase, 1 hr	13.2
0.5 mg/ml pancreatic lipase, 1 hr	120.
0.5 mg/ml chymotrypsin, 1 hr	91.
0.5 mg/ml trypsin, 1 hr	86.
pH 2.0, 1 hr	< 0.1
pH 12.0, 1 hr	4.15
1% Na deoxycholate *	0.39
1% Triton X100 *	139.
10^{-2} M NaCl *	19.2
10^{-1} M NaCl *	< 0.1
10^{-4} M TDF, 1 hr, pH 7.0	24.5

Except when indicated by * the indicated treatment was performed on membrane fractions *before* measurement of ACh binding in the standard conditions. When indicated by * the dialysis was done *in the presence* of the effector. Membrane fractions usually contain from 2 to 9 mg of protein per ml. The free concentration of ACh was 10^{-6} M.

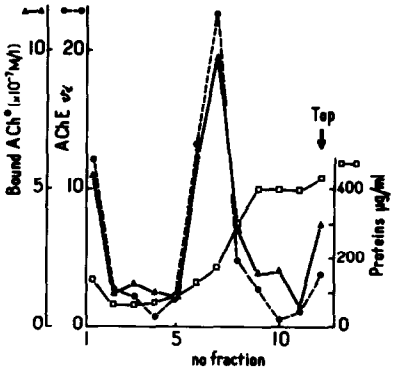

Fig. 2. Sedimentation in sucrose gradient of membrane fragments treated with 1% deoxycholate. Sodium deoxycholate is first added at room temperature for a few minutes to the suspension of membrane fragments in sucrose containing 9.2 mg protein per ml. The mixture is then dialysed against 1% deoxycholate in 10^{-3} M Na phosphate, pH 7.0, for 1 hr and added on top of a sucrose gradient stabilized in 10^{-3} M Na phosphate, pH 7.0, supplemented with 1% deoxycholate. The gradients are centrifuged for 4 hr at 50,000 rpm in a SW 65 rotor in a Beckman Spinco preparative ultracentrifuge. AChE and ACh binding in the presence of 10^{-5} M eserine are measured following the procedure described in section 2. Initial rates of acetylthiocholine hydrolysis (v_i) are expressed in mmoles/min/µl of fraction.

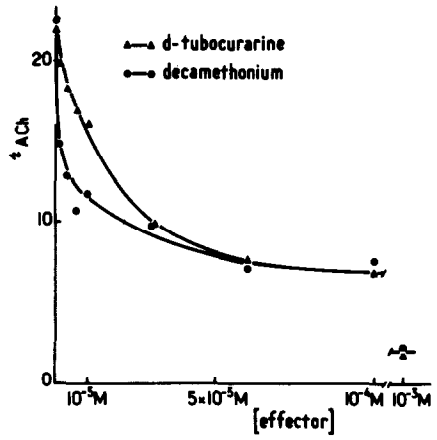

Fig. 3. Antagonism of ACh binding to pure AChE by d-tubocurarine and decamethonium. The free concentration of ACh in the absence of effector was 10^{-6} M. The concentration of enzyme was 1.2×10^{-3} M.

the binding data is not linear and does not extrapolate to a finite number of sites at infinite ligand concentration (fig. 1). Furthermore at high ACh concentrations the number of ACh molecules bound per molecule of AChE becomes remarkably large, values up to several hundred are found. This result is not accounted for simply by our present knowledge of AChE quaternary structure ($\alpha_2\beta_2$ dimer) [3]. The apparent number of ACh binding sites measured in these conditions is several orders of magnitude larger than the number of AChE protomers.

However, ACh bound to AChE is displaced by compounds pharmacologically active in the electroplax preparation [4], such as decamethonium, a depolarizing agent (or receptor activator), d-tubocurarine and flaxedil, two reversible receptor inhibitors (fig. 3). Preincubation of AChE with p-(trimethylammonium) benzene diazonium fluoroborate (TDF), an irreversible receptor inhibitor [5], is followed by a dramatic loss of its capacity to bind ACh. Furthermore, as shown in table 1 for NaCl, ACh binding is considerably reduced in the presence of high salt concentrations (10^{-1} M NaCl, 10^{-3} M $CaCl_2$ or $MgCl_2$) or of a negatively charged detergent (1% Na deoxycholate) but remains unchanged or slightly increases in the presence of a non ionic detergent (1‰ Triton X100). The binding of ACh in the presence of eserine seems thus to involve a special class of sites, distinct from AChE catalytic sites, which present a high affinity for curare-like agents. The positive charge of the ACh molecule is essential for the interaction of ACh with these sites.

Of importance is the observation that this unusual capacity for ACh binding appears to be a specific property of AChE. First of all, the total amount of ACh bound by the membrane fragments is completely accounted for by their content in AChE, estimated through an enzyme test (about 5% w/w of the total protein). Furthermore four unrelated proteins tested in the same conditions, serum albumin, β-galactosidase, ribonuclease, and catalase, do not show any significant binding of ACh.

Neither the physico-chemical interpretation nor the physiological significance of this unique property of AChE are yet clear. An attractive hypothesis relates it to the previously suggested function of AChE as the macromolecular receptor of ACh through *regulatory sites*, distinct from its catalytic centre, but specific for quaternary nitrogen-containing compounds [6]. However, the correlation of this molecular property of AChE to the *in vivo* pharmacological effects observed with the entire cell has not yet been done; in addition it is not excluded that other ACh binding proteins distinct from AChE might be present in small amounts in the extract and remain undetected in the present experimental approach.

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