PROPERTIES OF THE NICOTINIC ACETYLCHOLINE RECEPTOR MACROMOLECULE OF *ELECTROPHORUS ELECTRICUS*

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1. Introduction

The observation that neurotoxins from elapid venoms are powerful curarizing agents [1] provides a class of potent and specific reagents that can be used to purify nicotinic receptors. Thus, the isolation of such receptors is the subject of many investigations [2–5] but the nature and the characteristics of the molecule remain uncertain. We report here the isolation from the electric organ of the eel, *Electrophorus electricus*, of a protein based on its ability to bind specifically and with high affinity a neurotoxin from cobra venom. We describe some of the properties of this molecule and of its complexes with cobra toxin and pharmacologically active ligands.

2. Materials and methods

The main neurotoxic component from the venom of the cobra, *Naja naja siamensis* (supplied by Miami Serpentarium Inc., Florida), was purified and tritiated according to the technique of Cooper and Reich [6]. A specific activity of 3 Ci/mmole was obtained by this method. The preparation of the membrane fragments from the cephalic part of the electric organ was carried out following the technique of Changeux et al. [7]. Other methods are described in the figure legends.

3. Results

3.1. *Preparation of soluble extracts* [8]

A nonionic detergent, Tween 80 (10% w/v water) was added to the suspension of membrane fragments to a final concentration of 1%. The resulting solution was dialysed against 1 mM Tris-HCl, pH 7.4, at 4° for 5 hr, then centrifuged at 100,000 *g* for 30 min. The receptor activity was assayed in the supernatant.

3.2. *Assay for nicotinic receptor* [8]

An assay was developed from the observation [8] that the toxin, a strongly cationic molecule, is not adsorbed to DEAE-cellulose at pH near neutral whereas the receptor, an acidic protein, adsorbs to DEAE-cellulose, as does the toxin–receptor complex. When the material solubilized from membrane fragments is incubated with the tritiated cobra toxin and then filtered through discs of DEAE-cellulose paper, only those [^{3}H]toxin molecules complexed to receptor adsorb to the paper. These discs are dried and adsorbed toxin measured by scintillation counting using a toluene PPO-POPOP mixture.

3.3. *Purification by gel permeation chromatography*

Gel permeation chromatography on Sepharose 4B was performed with soluble extracts containing 5–6 mg/ml of protein. The profile in fig.1 shows that

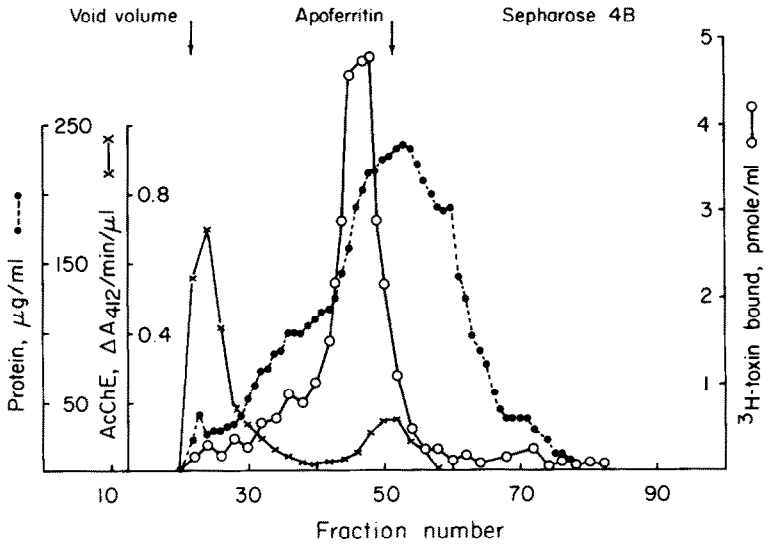

Fig. 1. Chromatography of 25 ml Tween 80 extract (see sect. 3.1.) on a column of Sepharose 4B (5.0 × 85 cm) at 4°. Eluent is 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.5% Tween 80. Fraction size is 24 ml. Binding activity was assayed as described (see sect. 3.2.) using 0.4 ml aliquots. Acetylcholinesterase activity was tested by the method of Ellman et al. [9]. The protein content was measured by the method of Lowry et al. [10] with bovine serum albumin as standard. Because of the presence of Tween 80, the samples were centrifuged before reading the absorbance at 750 nm.

a component of high molecular weight displays cobra toxin binding activity. This component emerges from the column very slightly ahead of apoferritin (M.W. 480,000) and is clearly separated from acetylcholinesterase. Based on a molecular weight of 500,000, the material in the tubes 45–48 is estimated to contain receptor at a purity of approx. 2% which represents a purification of five thousand fold from the starting material. Further purification to a high degree of purity can be accomplished by affinity chromatography, ion-exchange chromatography (DEAE-cellulose, hydroxylapatite) and gel permeation chromatography on Sepharose 6B [8].

3.4. *Kinetic and equilibrium studies*

These were performed on the material purified by gel permeation chromatography (fig.1, fractions 45 to 48) and concentrated to 0.75 mg/ml protein by vacuum dialysis. The binding activity for cobra toxin is stable for 2 months at 0° but is lost on freezing and thawing. The rate constant of toxin–receptor association (at conc. of 3×10^{-8} M and 1.2×10^{-9} M for toxin and receptor, respectively) is determined at 25° by using the DEAE paper assay. This gives a half-time of association of 140 sec which yields a k_1 of 1.67×10^5 M^{-1} sec^{-1}. The rate constant of toxin–receptor complex dissociation is measured as follows: the extract containing receptor is preincubated with the tritiated toxin for 1 hr at 25°, a condition in which free toxin, free receptor and complex remain stable and retain full activity. Then a three thousand fold exess of cold toxin is added and the concentration of the residual [^{3}H]toxin–receptor complex is measured as a function of time. Under these conditions, the complex does not dissociate completely, even after 2 days. There are two components in the dissociation curve, a rapid and a much slower one. The apparent fast component which accounts for 65% of the dissociation has a half-time of 140 min which yields a k_2 of 7.25×10^{-5} sec^{-1}. The dissociation constant calculated from the ratio k_2/k_1 of the rate constants is 4.3×10^{-10} M. This value is in close agreement with that (9.2×10^{-10} M) calculated separately from equilibrium data as shown in fig. 2.

The binding of [^{3}H]toxin to receptor is tempe-

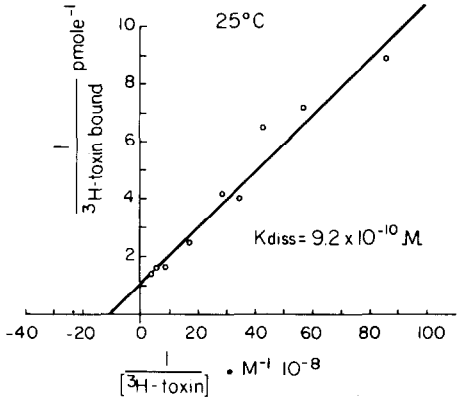

Fig. 2. Reciprocal plot of the binding of tritiated cobra toxin to partially (see sect. 3.4.) purified receptor. The ordinate shows the reciprocal of the [^{3}H]toxin bound to the receptor, the abscissa the reciprocal of the [^{3}H]toxin concentration. The reaction is performed in 4 ml final volume containing 130 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% Tween 80, 2.5×10^{-10}M receptor; bound toxin is determined after 80 min of incubation at 25°, using the DEAE paper assay. Each open circle represents the mean of two separate experiments. The straight line has been traced according to the data obtained with Cleland's computer program [11].

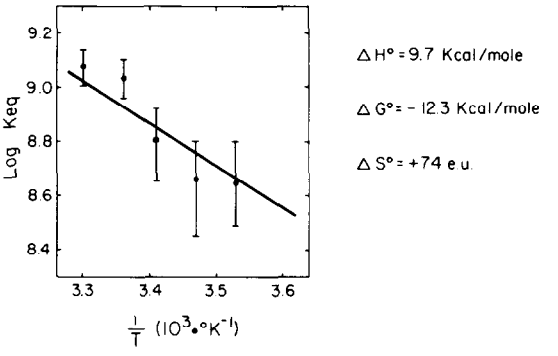

Fig. 3. Dependence on temperature of the equilibrium constant of the [^{3}H]toxin–receptor complex. The equilibrium constants are expressed as the reciprocal of the dissociation constants which have been calculated from data similar to that in fig. 2. The straight line and errors are traced according to the values given by computer analysis on Cleland's program [11].

rature dependent. It is stronger at 30° than at 10°. This dependence on temperature is clearly seen in an Arrhenius plot of the equilibrium constant as a function of temperature (fig.3). The data for the different temperatures have been obtained exactly as described in fig.2 for 25°. The ΔG° calculated from the equilibrium constant (1.1×10^9 M^{-1}) at 25° is −12.3 Kcal/mole. From the dependence of the equilibrium constant on temperature (fig.3) the ΔH° is 9.7 Kcal/mole. The entropy change at 25° is 74 e.u. The fact that the reaction is driven by a large entropy change implies that increasing disorder of the receptor and/or toxin accompanies complex formation. It should be noted that Raftery et al. [5] reported that crude membrane fractions of the same origin have lower affinity for α-bungarotoxin than the one we observed for solubilized receptor and cobra toxin.

3.5. *Pharmacological data*

One major question of interest related to the receptor concerns its interaction with cholinergic agonists and antagonists. Competition studies between drugs and toxin have been performed on the material purified by gel permeation chromatography (fig.1, fractions 45 to 48). The dissociation constants were calculated from equilibrium data obtained after 80 min of incubation of receptor, [^{3}H]toxin and cholinergic drugs at different concentrations. Muscarinic agents and cholinesterase inhibitors are inert whereas nicotinic agonists and antagonists compete effectively. The binding pattern of nicotinic agents vary widely: for example nicotine, dimethyltubocurarine and alloferin which behave as competive inhibitors, of the toxin through a wide range of concentrations, show a typical relationship for hyperbolic competitive inhibition as defined by Cleland for enzymes [13]. Different patterns of inhibition are given by hexamethonium, decamethonium and carbamylcholine, respectively.

4. Discussion

Although putative acetylcholine receptor macromolecules isolated from different sources have been identified respectively as protein, acidic mucopolysaccharide, nucleoprotein, phospholipoprotein or lipoprotein, the nature of the pure receptor is still unknown. The

molecule we have isolated behaves in all respects like a protein as shown by direct chemical analysis (Folin), sensitivity to trypsin and pronase and insensitivity to phospholipase C and other lytic enzymes. Certain discrepancies exist in the literature regarding the molecular weight of the receptor. While the properties of our preparation on gel permeation chromatography resemble those described by others [3, 5], we cannot make an assignment of molecular weight with confidence since the final steps in the purification procedures are so far reproducible only in the nanomole range of receptor concentration and also because several different forms of aggregation have been encountered. The behaviour of hydrophobic proteins on gel permeation chromatography and centrifugation in sucrose gradients cannot serve as a means for estimating molecular weight because the reference markers used for calibrating these procedures are unrelated hydrophobic proteins whose interaction with detergents cannot be compared with that of the receptor. The equilibrium of the toxin–receptor interaction, which demonstrates the reversibility of complex formation, provides a sensitive probe for exploring the binding of pharmacologically important ligands to the receptor. Observations of this kind yield a detailed model of drug–receptor interactions that is presented in full elsewhere [12].

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