COMPARISON BETWEEN THE AFFINITIES FOR REVERSIBLE CHOLINERGIC LIGANDS OF A PURIFIED AND MEMBRANE BOUND STATE OF THE ACETYLCHOLINE-RECEPTOR PROTEIN FROM *ELECTROPHORUS ELECTRICUS*

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1. Introduction

The functional properties of the cholinergic receptor protein from *Electrophorus electricus* can now be investigated at three distinct levels: the cell level, with the electroplax preparation [1], the membrane level, with the chemically excitable microsacs [2], the molecular level, with the recently purified receptor protein in solution [3]. Comparison of the dose-response curves recorded *in vivo* with those measured *in vitro* showed that, upon "reduction" from the cell level to the membrane level, the system showed no significant change in its pharmacological properties: clearcut agreement was obtained between the binding curves of cholinergic ligands to the excitable microsacs and their dose-response curves to the same ligands [2]. This last finding was recently confirmed by Weber et al. by an entirely different method [4].

In this letter, we present the results of binding experiments performed with the purified receptor protein (AcCh–R) and several cholinergic ligands, in the presence and in the absence of detergents. Under all the conditions tested the affinity of the purified protein for three cholinergic agonists exceeds by about 10–50 times that of the membrane-bound receptor, for the same ligands, whereas the affinity for three antagonists remains about the same. Several interpretations of this phenomenon are proposed.

2. Materials and methods

2.1. Purification of the AcCh–R

Purification and concentration procedures were basically the same as previously reported [3]. We used an affinity column of 250 ml yielding 7 to 9 mg of protein and 30 nanomoles of [^{3}H]α-toxin binding sites in about 3 ml of 0.01 M Tris pH 7.4, 1.0 M NaCl, 1% Triton X-100 (v:v) and 0.02% Na-azide (w:v).

2.2. Exchange of detergent

Replacement of readily exchangeable Triton X-100 by Na-cholate was achieved by sucrose density gradient centrifugation [5]. One to 2 mg of purified receptor in 0.4 to 0.8 ml of 0.010 M Tris pH 7.4, 0.10 M NaCl, 1% *Triton X-100*, 0.02% Na-azide was layered on top of a 10 ml gradient consisting in 5 to 20% (w:v) sucrose in 0.010 M Tris pH 7.4, 0.10 M NaCl, 0.5% *Na-cholate* (w:v) and 0.02% Na-azide. The sample was centrifuged at 3.9×10^4 rpm for 14 to 16 hr at 4° in a SW 41 Ti rotor of a Beckman (model L3-50 or L2-65B) ultracentrifuge. Fractions of 0.4 ml were collected with the help of a Büchler piercing unit. Protein was assayed in 0.05 ml of each fraction by the method of Lowry et al. [6] using bovine serum albumin as the standard. Receptor activity was assayed in 1 µl of a 10-fold dilution of each fraction in "helper" [3] using our Millipore assay. A typical sedimentation profile is shown in fig. 1. Fractions with the highest specific

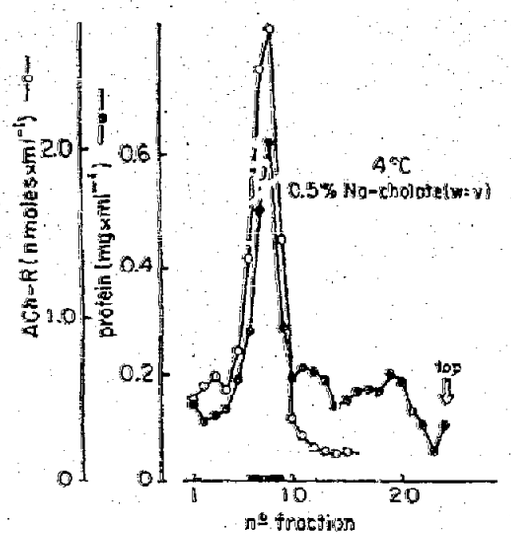

Fig. 1. Sedimentation profile of the purified acetylcholine-receptor after affinity chromatography (details in Materials and methods, sect 2.2).

activity (4.5 ± 0.5 μmoles of [^{3}H]α-toxin binding sites per g protein were pooled (horizontal bar))*.

2.3. Removal of detergent

Unbound Na-cholate was separated from protein by gel filtration through Sephadex G-75. The sample (0.7 mg of protein in 2 ml of buffer) was centrifuged as described above, except that the sucrose gradient(s) contained, in addition to 0.5% Na-cholate, traces of Na-[^{14}C]cholate (2.7×10^5 cpm). The column (40 ml) was equilibrated in 0.002 M sodium phosphate buffer pH 7.0, 0.15 M NaCl at 4° and the flow rate was 8 ml/hr. Fractions of 1.5 ml were collected. Absorbances were measured at 280 nm. Radioactivity was measured in a Intertechnique ABAC SL40 liquid scintillation counter after dilution of 0.10 ml of every fraction in 10 ml of Bray's solution. A typical gel filtration profile is shown in fig. 2

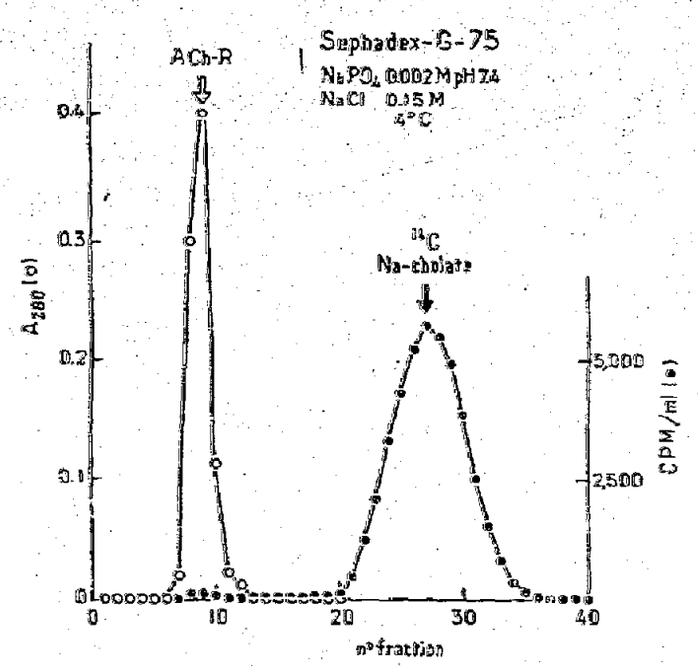

Fig. 2. Separation between purified AcCh–R and unbound Na-cholate by gel filtration through Sephadex G-75 (details in Materials and methods, sect 2.3).

2.4. Equilibrium dialysis

Equilibrium dialysis experiments were carried out essentially as recommended by Gilbert and Müller-Hill [8], with [^{3}H]decamethonium as the radioactive ligand in 0.0015 M Na-phosphate pH 7.0, 0.16 M NaCl, 0.005 M KCl, 0.002 M $MgCl_2$, 0.002 M $CaCl_2$, 0.02% Na-azide. When needed, dialysis buffer contained either Triton X-100 (1%, v:v) or Na-cholate (0.5%, w:v). Routinely, 0.2 ml of the solution of purified receptor (14 to 50 μg of protein or 50 to 270 pmoles of [^{3}H]α-toxin binding sites) in a bag was dialyzed against 20 ml of buffer in a test tube, for 15 to 17 hr at 4°, on a rocking shaker. After equilibration, radioactivity was measured in 0.05 ml of both the bag content and dialysis buffer. Since diffusion of d-tubocurarine (dTC) through the membrane is particularly slow, dTC was added, prior to dialysis at the desired concentration both *inside* and outside the bag.

2.5. Biological activity of [^{3}H]decamethonium

Exact determination of the number of decamethonium binding site and of binding affinity requires a precise estimate of the concentration of pharmacologically active decamethonium. Dose-response curves to

* This material gives 1) a single band by disc gel electrophoresis in the presence of 1% cholate or 1% emulphogen, 2) a single peak with constant specific activity by sucrose gradient centrifugation, 3) a homogenous population of particles of 90 Å diameter with an electron dense center by negative staining. The precise stoichiometry of toxin binding sites per mass of protein is not yet firmly established because of uncertainties on the exact specific activity on the tritiated α-toxin and on the estimation of protein concentration.

[^{3}H]decamethonium were thus recorded with the isolated electroplax preparation by the method of Higman et al. [7] and compared to the dose-response curves to unlabelled decamethonium from various origins. The pharmacological activity of tritiated decamethonium was (0.65 ± 0.05) times that of unlabelled decamethonium. Concentrations of [^{3}H]decamethonium were thus always corrected and expressed in terms of *biologically active* decamethonium molecules.

2.6. Chemical sources

Triton X-100: Calbiochem; Na-cholate: Schuchardt; Sephadex G-75: Pharmacia; decamethonium bromide, carbamylcholine chloride, d-tubocurarine chloride and hexamethonium bromide: K & K Laboratories; phenyltrimethylammonium chloride: Eastman Organic Chemicals; gallamine triethiodide (flaxedil): S.P.E.C.I.A.; Na-[^{14}C]cholate (60 Ci/mole) and [^{3}H]decamethonium chloride (400 Ci/mole): the Radiochemical Centre, Amersham. Chemicals were used without any additional purification.

3. Results

The yield of our purification procedure is high enough to allow a study of the binding of cholinergic ligands to the purified receptor protein (specific activity ranging from 3.0 to 6.0 μmoles [^{3}H]α-toxin binding sites per g protein) by the simple method of equilibrium dialysis of Gilbert and Müller-Hill [8]. For instance, under the experimental conditions given in Methods and in the presence of 10^{-7} M free decamethonium, there were 2.25 times more counts per unit volume inside than outside the bags at equilibrium. In addition no marked inactivation occurred during the experiment and no artefactual binding of [^{3}H]decamethonium to glass or dialysis bags was ever noticed.

Fig. 3. shows the binding curve of [^{3}H]decamethonium to AcCh–R as a function of concentration of radioactive ligand. In this experiment the neutral detergent Triton X-100 was present both inside and outside the bag. Treatment of the purified AcCh–R with an excess of *Naja nigricollis* α-toxin prior to dialysis *completely* abolishes binding of [^{3}H]decamethonium. Analysis of the binding data by double

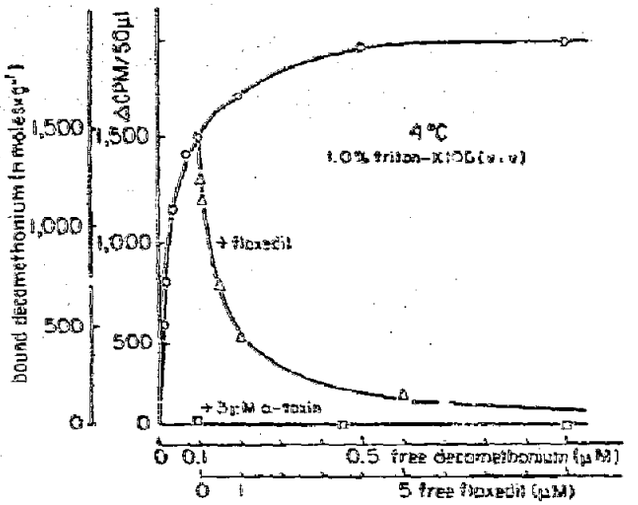

Fig. 3. Binding of [^{3}H]decamethonium to purified AcCh–R in the presence of 1% Triton X-100 (○—○—○) and its complete antagonism by α-toxin from *Naja nigricollis* venom (□—□—□). On the same graph inhibition of bound [^{3}H]decamethonium at a fixed concentration (0.1 μM) by increasing amounts of gallamine (flaxedil) is shown (△—△—△). The concentration of decamethonium are *not corrected* for actual pharmacological activity of [^{3}H]decamethonium.

reciprocal plot indicates that, within the range of concentration explored (up to 1 μM decamethonium) and within the limits of accuracy of our measurements the binding curve can be fitted by a hyperbola for, at least, 75% of the sites. Half saturation occurred at 2.1 ± 1 × 10^{-8} M decamethonium. The number of sites for decamethonium was 2.1 μmole/g protein, a number which is close enough to that of [^{3}H]α-toxin binding sites: 3.3 ± 0.5 μmole/g protein measured by the Millipore assay [3].

The same graph shows that a known cholinergic antagonist, flaxedil, *completely* displaces [^{3}H]decamethonium from its binding site on the purified AcCh–R protein. Similar competition experiments were made for a variety of cholinergic agonists and antagonists. The relevant dissociation constants (K_D) estimated from these curves on the basis of a competitive interaction between cholinergic ligands are given in the table.

The values obtained in the *presence* of 1% Triton X-100 do not differ from those obtained in the *presence* of 0.5% Na-cholate after exchanging Triton X-100 by cholate as described in Methods (table 1). Removal of Na-cholate by filtration on Sephadex G-75 and equilibrium dialysis in the *absence* of detergent is not

Table 1

Comparison between the dissociation constants of the reversible complexes of various cholinergic ligands and the AcCh–R protein in a membrane bound and purified state.

	Membrane bound AcCh–R		Purified AcCh–R		
	Binding of [^{3}H]deca to mirosacs	Protection against [^{3}H]α-toxin binding	1% Triton X-100	1% Na-cholate	No free detergent
	K_D (mole × ℓ^{-1}) 22°	K_D (mole × ℓ^{-1}) 22°	K_D (mole × ℓ^{-1}) 4°		
Agonists					
Decamethonium	1.3×10^{-6} a	0.8×10^{-6} b	2.1×10^{-8}	2.1×10^{-8}	4.3×10^{-8}
Carbamylcholine	4.0×10^{-5} a	2.2×10^{-5} b	1.9×10^{-6}	2.4×10^{-6}	–
Phenyltrimethyl-ammonium	(2.0×10^{-5}) c	–	1.2×10^{-6}	1.0×10^{-6}	1.2×10^{-5}
Antagonists:					
d-Tubocurarine	2.0×10^{-7} a	1.7×10^{-7} b	3.9×10^{-7}	3.7×10^{-7}	–
Gallamine (flaxedil)	4.0×10^{-7} a	4.4×10^{-7} b	1.3×10^{-7}	1.2×10^{-7}	1.3×10^{-7}
Hexamethonium	–	6.1×10^{-5} b	6.2×10^{-5}	5.7×10^{-5}	–

Values under "membrane bound AcCh–R" are from: [a] Kasai and Changeux [2]; [b] Weber et al. [4] and Weber and Changeux [10]; in [c] is given the *apparent* dissociation constant for phenyltrimethyl ammonium measured by following the *in vitro* response of excitable microsacs by Kasai and Changeux [2]. Binding of [^{3}H]decamethonium was measured directly; that of the other cholinergic ligands by competition against [^{3}H]decamethonium binding; K_D for this last category of compounds was calculated from the equation $K_D = I_{50} \cdot K_{Deca}/K_{Deca} + [Deca]$ where I_{50} is the concentration of ligand which reduces by half the amount of [^{14}C]decamethonium bound, [Deca] the free concentration of decamethonium and K_{Deca} its dissociation constant.

accompanied by any dramatic change of the dissociation constants. However, after Sephadex filtration, 15 to 17% of Na-cholate on a weight to weight basis remains bound to the AcCh–R protein. Under these conditions the purified receptor protein no longer precipitates in the absence of detergent except when the "helper" fraction present in crude extracts is added.

In earlier experiments done with crude extracts [9], the affinities found for decamethonium and other agonists were somewhat lower than those measured with the purified material. We presently know that under the conditions formerly used (crude extracts containing the "helper" fraction after precipitation of deoxycholate by $MgCl_2$, dialysis against Ringer's solution *without* detergent) a significant reaggregation of the receptor protein took place. Binding of [^{3}H]decamethonium to crude extracts was therefore re-investigated after solubilisation by Triton X-100 and with Triton X-100 present both inside and outside the bags during dialysis. We found that half saturation of the sites from which decamethonium is displaced by the α-toxin occurs at 2.5×10^{-8} M free decamethonium, a value very close to that found with the purified protein. The number of the decamethonium binding sites is 14 nmoles/g protein, a value close to that of [^{3}H]α-toxin binding sites present in the same extracts: 16 nmoles/g protein. The dissociation constant for flaxedil and phenyltrimethyl ammonium measured by competition against [^{14}C]decamethonium binding were, respectively, 1.3×10^{-7} M and 1.10×10^{-6} M.

4. Discussion

Decamethonium, a known cholinergic agonist, binds with a high affinity to the purified receptor protein. Decamethonium bound to the AcCh–R is *completely* displaced by *N. nigricollis* α-toxin and flaxedil which both act *in vivo* as potent nicotinic antagonists. This result confirms our earlier findings on membrane fragments [2] and crude extracts [9] and demonstrates the validity of our assay for the cholinergic receptor site [9].

In the table we compare the binding data obtained with the purified receptor protein and with membrane fragments. The data for the membrane-bound receptor protein come from two independent series of experiments. In a first one, direct binding of [^{14}C]deca-

methonium to membrane fragments was measured by a simple centrifugation assay [2]. The fraction of [^{14}C]decamethonium bound to the membrane fragments *and* displaced by the α-toxin was considered as specifically associated with the cholinergic receptor site. In a second one, *initial rates* of [^{3}H]α-toxin binding were measured at various concentrations of cholinergic ligands [4, 10]. It was shown that the protection curves could be taken as binding curves of the cholinergic effector [4, 10]. Interestingly, the two series of experiments done with membrane fragments give convergent results but a significant difference appears between membrane-bound and purified receptor. The affinity of the three considered agonists (acetylcholine can be added to this list) is one to two orders of magnitude larger with the purified protein than with the membrane fragments. On the other hand no significant difference is seen with the three antagonists. The same results are obtained in the presence of a neutral or negatively charged detergent and in the "absence" of detergent. Recently, Franklin and Potter [11] have noticed a 10-fold *decrease* in the affinity for carbamylcholine upon solubilisation of the receptor protein from *Torpedo* membrane fragments. The effect is in a direction opposite to the one we find but might be relevant to the fact that *Torpedo* and *Electrophorus* receptors show quite different binding properties [10].

Several interpretations can be proposed for the differences of affinity observed between membrane bound and purified receptor from *Electrophorus*: 1) In the membrane fragments and with the purified protein we follow binding to sites carried by different species of receptor protein or iso-receptors. For instance, the affinity chromatography might select a fraction of molecules which present a high affinity for cholinergic agonists and carry only a small proportion of the sites measured in the membrane fragments. Relevant to this hypothesis is the fact that the yield of our purifications never exceeds 50% and that the receptor sites present in the membrane fragments show a minor but significant heterogeneity [10]. The membrane fragments used for binding studies and for solubilisation of the receptor protein might contain different ratios of extrasynaptic and subsynaptic receptor sites although we do not know if these two classes of receptor possess the same binding properties. 2) The receptor protein possesses two distinct classes of sites for agonists with marked differences of affinities [12]. In the binding studies with the purified protein, the low affinity sites would remain undetected. 3) The same sites are involved but carried by two different conformations of the receptor protein. Solubilisation stabilises a conformation with high affinity for agonists.

Interpretations 1 and 2 are made unlikely by the fact that i) solubilisation of the membrane fragments is not accompanied by a significant loss of [^{3}H]α-toxin binding sites [3], ii) the ratio of [^{3}H]decamethonium to [^{3}H]α-toxin binding sites remains close to 1 both in crude extracts and after purification.

The third interpretation fits with the hypothesis [13, 14, 17] that the cholinergic receptor protein is a regulatory protein. The solubilisation by detergents would release a *membrane constraint* created by either membrane lipids or proteins or by both and stabilise the molecule in an "active" or permeable conformation. This conformation would be the same for all the agonists tested, which, incidently, would favor a "two-states" rather than an "induced-fit" model [15]. The limited changes of affinities for the antagonists would be caused by their *non-exclusive* binding [16, 17] to both the "active" and "resting" conformations whilst the agonists would bind exclusively to the "active" state. Release of the membrane constraint would then become analogous to the release of the quaternary constraint, found with regulatory enzymes under conditions of "desensitization" [18]. Experiments are in progress to test these various alternatives.

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