Biochemical Pharmacology, Vol. 22, pp. 3145-3150. Pergamon Press, 1973. Printed in Great Britain.

COOPERATIVITIES IN THE BINDING OF ACETYLCHOLINE TO ITS RECEPTOR

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(Received 23 August 1973; Accepted 8 September 1973)

Communicated by: Alan C. Sartorelli

Positive cooperative interactions have been detected in concentration-response curves obtained from the application of activators to skeletal and smooth muscles (1) and the electroplax of the electric eel, Electrophorus electricus (2,3). On the other hand, there are no reports of negative cooperativity, unless we consider neuromuscular desensitization (4,5) as such. Since these physiological responses represent the end result of several molecular events starting with ligand binding to the receptor, it is difficult to conclude from such data whether or not the cooperative effects occur at the binding step.

The availability of a purified acetylcholine receptor (AChR), whose acetylcholine (ACh) binding is blocked by nicotinic cholinergic drugs and α-bungarotoxin (6), provides the tool with which one can study the details of the transmitter-receptor interactions. We had previously observed two affinities in the binding of ^{3}H-ACh to AChR of the electric ray, Torpedo marmorata whether membrane-bound (7), Lubrol-solubilized (8) or purified (6). In this report, we present the results on the binding to the purified AChR of ^{3}H-ACh at 28 concentrations, so that the increments are greatly reduced and the range expanded; thus allowing the detection of further changes in the binding data. Multiple affinities are observed as well as both positive and possibly negative cooperativities, and the physiological relevance of these homotropic effects are discussed.

MATERIALS AND METHODS

The AChR preparation used is purified (7.8 nmoles ACh-bound/mg protein) by affinity adsorption (6) from the Triton X-100 extract of the lyophilized pellet of 12,000 x g of Torpedo marmorata electroplax (9). Two preparations of ^{3}H-ACh were used, a 49.5 mCi/mmole (from New England Nuclear) used for the concentration range of 0.01 to 5 μM and a 290 mCi/mmole (from Amersham Searle) for the low ACh concentrations (0.0007 to 0.01 μM). Their radiochemical purity was periodically checked by thin layer chromatography (10).

Binding of ^{3}H-ACh was measured by equilibrium dialysis as previously described (11). We inhibited the minute amount of ACh-esterase still present in this highly purified AChR preparation (one catalytic site for every 20,000 ^{3}H-ACh binding sites (6)), by preincubation with Tatram (O,O-diethyl S-diethyl-aminoethyl phosphorothiolate) at 10 μM for 30 min. before start of equilibrium dialysis and by having 1 μM of it in the equilibrium bath. Only above 0.1 mM does Tatram block binding of cholinergic ligands to this AChR (12). After equilibrium, three samples were taken from bath and bag contents at each ^{3}H-ACh concentration used. Excess radioactivity in bag samples represented bound ACh.

RESULTS

Binding of ^{3}H-ACh to the purified AChR deviates from a Langmuir isotherm, and in fact two inflections are consistently observed in a linear plot (Fig. 1).

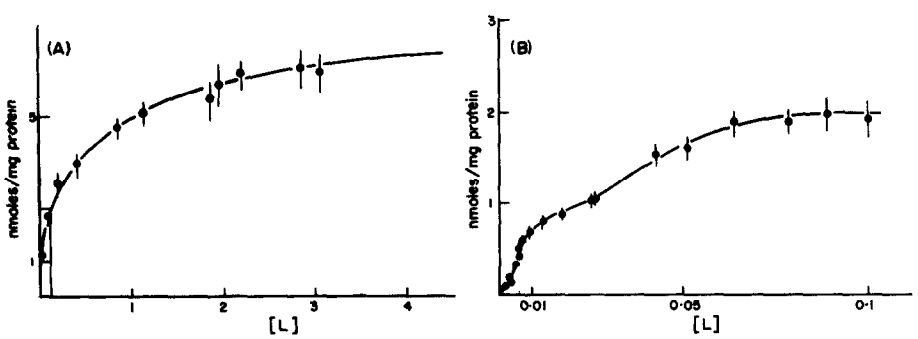

Fig. 1 Binding of ^{3}H-ACh to the purified AChR. [L], concentration of ^{3}H-ACh in μM. Each point is the mean of four experiments and the vertical line is the standard error. A. Binding at concentrations 0.01 to 4 μM. B. Binding at concentrations 0.0007 to 0.1 μM i.e. portion within inset of Fig. A.

At very low ^{3}H-ACh concentrations (0.0007 to .01 μM), the binding data appear as an upward curved line in a Scatchard plot (Fig. 2). When the maximum binding sites involved in this range are obtained by extrapolation, and the data plotted on a Hill plot, the average Hill coefficient is 1.6 ± 0.2. These results suggest positive cooperativity. Binding of ^{3}H-ACh to the purified AChR at higher concentrations (0.5 - 5 μM) is also non-linear in a Scatchard plot (Fig. 3), but deviates in the opposite direction. The Hill coefficient is 0.5 ± 0.1, which suggests possible negative cooperativity.

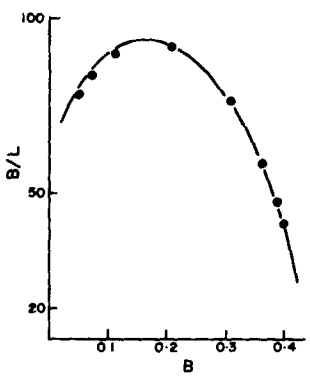

Fig. 2 Scatchard plot of the amount of ^{3}H-ACh bound to AChR at low ACh concentrations. B, Amount bound in nmoles per mg protein; L, concentration of ^{3}H-ACh in μM.

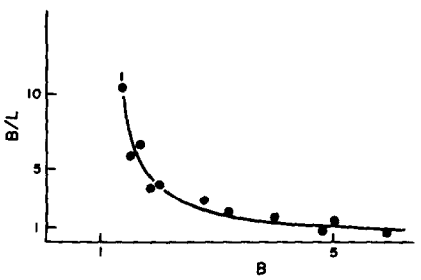

Fig. 3 Scatchard plot of the amount of ^{3}H-ACh bound to AChR at high ACh concentrations. B and L as in Fig. 2.

DISCUSSION

Binding of ACh to the purified AChR is complex and occurs with several affinities that are detectable at equilibrium, and dependent on the ACh concentration used. The observed inflections in the binding curve (Fig. 1a,b) and the detected cooperativities (Fig. 2,3) suggest that this purified AChR is oligomeric, with multiple ACh-binding sites per molecule. This agrees with the estimated molecular weight of this purified AChR as being above 300,000 (possibly half a million (6)) and of the subunit carrying one ACh-binding site ranging between 83,000 and 112,000 (6). It is also in line with electron micrographs of

the negatively stained AChR, which appears as a globular doughnut with three to six subunits (13, also our own unpublished data). These findings suggest that Lineweaver-Burk or Scatchard type plots are inadequate for the analysis of such binding. It also appears that the affinities of a ligand for AChR, determined indirectly by its blockade of the binding of a radiolabeled ligand (e.g. ACh, decamethonium or neurotoxins from snake venoms), would depend largely upon the portion of the binding curve selected and the concentration of competing ligand used.

A novel observation is the positive cooperativity in the binding of ^{3}H-ACh to AChR at very low ACh concentrations (Fig. 2). This phenomenon is not restricted to ACh, we have also observed it in the binding of ^{3}H-decamethonium to this preparation in the absence of any Tetram (unpublished). The Hill coefficient of 1.6 is similar to those calculated from dosage-response curves of the monocellular preparation of eel electroplax (1.8-2 (2) or 2 (3)) or skeletal and smooth muscles (1.5-2 (1)).

The lower affinities observed at higher ligand concentrations may be a result of ^{3}H-ACh binding to different non-interacting sites on the same or different forms of AChR or to negative cooperativity between two or more sites. This may be related to the phenomenon of desensitization observed *in situ* when high dosages of activators are applied to skeletal neuromuscular junctions (4) and *Torpedo* electroplax (14). The exhibition of both positive and negative cooperativities in the binding of a single protein is not unique and has been observed in the binding of nicotinamide-adenine dinucleotide to yeast glyceraldehyde 3-phosphate dehydrogenase (15).

We realize that the purified AChR preparation studied still contains a small amount of detergent (Triton X-100 at 0.007%) (6), and is treated with 10 μM Tetram so as to inhibit the minute amount of acetylcholinesterase still present. These factors plus others that affect the binding e.g. the buffer employed and the state of aggregation of AChR, may be influencing the observed cooperativities and may not reflect the situation *in vivo*. Such homotropic

cooperativities, if existing in vivo, would be advantageous in amplifying the effect of low concentrations of ACh and in desensitizing AChR at high ACh concentrations. These positive and negative cooperativities would provide the mechanisms of the regulatory function of the AChR.

The various observed affinities in the binding of ^{3}H-ACh to this purified AChR cannot be accurately determined until a special curve-fitting program is obtained; and this is currently in progress. It will be feasible then to relate such data to the allosteric (16,17) or induced fit models (18). The use of fast techniques, such as stopped flow, temperature jump or dynamic sedimentation will permit the detection of conformational changes in the receptor molecule.

This research was partially financed by U.S. Public Health Service grant NS 09144.

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