IDENTIFICATION OF A CALCIUM-BINDING SUBUNIT OF THE ACETYLCHOLINE RECEPTOR

Helga Rübsamen, Mary Montgomery and George P. Hess†
Section of Biochemistry

and

Amira T. Eldefrawi* and Mohyee E. Eldefrawi†*
Section of Neurobiology

Cornell University, Ithaca, N.Y. 14853

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Summary: Four subunits of the acetylcholine receptor molecule, obtained from the electric organ of Torpedo ocellata, have been isolated using polyacrylamide gel electrophoresis, and assayed by titration with a fluorescent lanthanide, terbium, and by affinity-labeling with p-(N-maleimido)benzyl [trimethyl-^{3}H] ammonium iodide. The site with which the activator-analogue affinity label reacts, as well as the terbium-binding sites, are mainly associated with the smallest of the subunits of an apparent molecular weight of 40,000. Calcium competes with terbium for these binding sites. The affinity for terbium is the same in the intact molecule as in the subunit ($K_{Tb} \simeq 19 \pm 1$ μM), but the affinity for calcium decreases by a factor of 4 ($K_{Ca} \simeq 4$ mM) in the subunit. Hydrolysis of the receptor, catalyzed by trypsin and chymotrypsin, to peptides with an apparent molecular weight of 8000 or less, does not affect the terbium-binding sites. These experiments indicate that the binding sites for neural activators and for calcium are associated with the same subunit, and that the terbium- and calcium-binding sites reflect structural properties of the polypeptide chain rather than the three-dimensional structure of the protein.

Experiments with nerve axons (1,2) and electroplax (3) have shown that Ca^{2+} ions strongly influence the electrical behavior of excitable membranes, presumably by regulating their permeability to inorganic ions (4,5). The purification of acetylcholine receptor proteins by a number of laboratories (e.g. 6-12) has made it possible to study one type of such interactions at a molecular level. In investigations using acetylcholine receptor-rich membrane fragments from Torpedo marmorata an increased affinity of the receptor for cholinergic ligands in the presence of Ca^{2+} was observed (13). When the purified receptor protein of T. californica was studied, it was found that Ca^{2+} competed with acetylcholine and a fluorescent cholinergic ligand (14,15). Recently, we have shown (16) that Tb^{3+}

†To whom reprint requests and correspondence should be addressed.
*Present address: Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Md. 21201.

competes with Ca^{2+} for receptor binding sites, and that both are displaced from these sites by activators, but not inhibitors, of cholinergic transmission. These findings are in harmony with previous experiments (17-20) which suggested that the membrane-bound acetylcholine receptor has separate binding sites for activators and inhibitors of neural transmission. Thus the possibility arises that displacement of Ca^{2+} from the receptor by activators is an initial event in the formation of ion channels which are responsible for an increase in the permeability of chemically excitable membranes to Na^+ and K^+.

In order to investigate further the rôle Ca^{2+} plays in acetylcholine receptor-mediated permeability changes, we are now attempting to identify the Ca^{2+}-binding sites on the subunits of the acetylcholine receptor. The acetylcholine receptors obtained from Electrophorus electricus and from Torpedo sp. have been characterized by SDS polyacrylamide gel electrophoresis in a number of laboratories (11, 21-23), and in addition to other subunits a major band due to a polypeptide of molecular weight between 39,000 and 48,000 has been detected. In this communication we report the purification of subunits of the acetylcholine receptor molecule isolated from T. ocellata, and the use of a fluorescent lanthanide, Tb^{3+}, which we have employed previously to characterize the Ca^{2+}-binding sites of the intact molecule, to study the Ca^{2+}-binding sites of the subunits. The subunit associated with the binding site for activators was identified by using the affinity label developed by Karlin et al. (24), p-(N-maleimido)benzyl [trimethyl-^{3}H] ammonium iodide ([^{3}H]-MBTA).

MATERIALS AND METHODS The acetylcholine receptor was purified from the electric organ of T. ocellata (obtained from the Mediterranean near Alexandria, Egypt) in EDTA as previously described (7,14). It bound 10 nmoles of acetylcholine or α-bungarotoxin per mg protein (25,26). The purified receptor was lyophilized and then denatured by treating about 600 µg with 200 µl of 2% SDS, 2% β-mercaptoethanol, 10% sucrose, for 2 h at 40°C. 600 µg of denatured receptor were applied to each gel slab. Gel slabs (0.3 cm x 10.3 cm x 8.7 cm) were made using 6.4% acrylamide, 0.2% N,N-methylene-bisacrylamide, 0.5% SDS (all from Biorad), 0.3 M Tris, 0.24% ammonium persulfate, 0.04% N,N,N',N',-tetra methylenediamine (Eastman), pH 8. The subunits were separated at 20°C, 30 mA/slab, using the following buffers: 6.32 g Tris, 3.94 g glycine, 0.3 g SDS per liter, pH 8.9. SDS was omitted from the lower buffer. At the end of a run (3 h), the gels were laid on an ice-cold glass plate and cut into slices 0.45 cm thick from which the subunits were recovered electrophoretically. The subunits were then dialyzed against a 10-fold excess of 2 mM Pipes buffer containing 0.03% Triton X-100, the

pH of which was adjusted to 6.5 using Tris (Sigma) base. The dialysis solution was exchanged 9 times at intervals of 4 h or more. In order to completely remove inorganic ions from the proteins, dialysis was done in nalgene bottles and the buffer was made and stored in nalgene containers. The fractions were then poured into nalgene test tubes and stored at 1°C. The volume of each protein fraction was adjusted to 1.5 ml using Pipes buffer. In a micro-cuvette, 100 µl of each fraction were diluted with 100 µl of the Pipes buffer. 10 µl of a 10^{-3} M Tb^{3+} solution were added in steps of 3 and 7 µl. Fluorescence was measured after each addition as described before (16). Protein concentration was determined according to the method of Lowry et al. (27). The values for Tb^{3+} fluorescence (at 50 µM Tb) and for protein concentrations were corrected for readings obtained from a blank gel run in parallel that had not been loaded with receptor. Slices of the gels cut from both edges of the gel from the top to the dye front were fixed and stained in 7.5% trichloroacetic acid, 50% methanol, 0.025% Coomassie Brilliant Blue, overnight at 20°C, and destained by diffusion in 7.5% acetic acid. The gels were scanned in a Gilford spectrometer at 560 nm.

Synthesis of the affinity label, [^{3}H]-MBTA (300 mCi/mmole), and the labeling of the receptor were performed essentially as described (9,24,28) except that the reaction time with the label was 10 min. After labeling the receptor was denatured and the subunits were separated on an analytical SDS-polyacrylamide gel of the same composition as the preparative slabs. The gels were stained for proteins, scanned as described above and cut into slices 2 mm thick. Radioactivity in the slices was determined as described (9).

RESULTS AND DISCUSSION Scatchard (29) plots of the binding isotherms of the intact acetylcholine receptor (●) and after denaturation with SDS and subsequent dialysis (o) are shown in Figure 1. The ordinate intercepts give $\Delta F_p(max)$, the maximum observed fluorescence intensity per mg receptor protein, when all Tb^{3+}-binding sites are occupied. The slope of the lines is proportional to the apparent dissociation constant of the Tb^{3+}-binding sites, K_{Tb}. The linearity of the data is consistent with homogeneous, independent Tb^{3+}-binding sites. The lines are essentially parallel, indicating that the value of K_{Tb} is similar whether or not the receptor is treated with SDS (intact molecule, 25 $\pm$ 1 µM; SDS-treated, 24 $\pm$ 1 µM). The similar ordinate intercepts for the native and SDS-treated receptor indicate that the Tb^{3+}-binding sites remain intact in the denaturation procedure. In other experiments the receptor was broken down by enzymic hydrolysis with trypsin and chymotrypsin to peptides of molecular weight 8000 or less as determined by polyacrylamide gel electrophoresis. The fluorescence of bound Tb^{3+} per mg protein at saturation, $\Delta F_p(max)$, is the same as in the intact molecule as shown by the ordinate intercept of the data (□) in Figure 1. We also observe close agreement between the dissociation constants for Tb^{3+}, which are given by the slopes of the lines. The Tb^{3+}-binding sites seem, there-

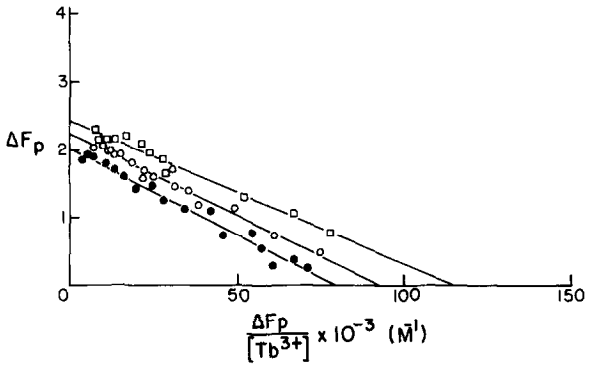
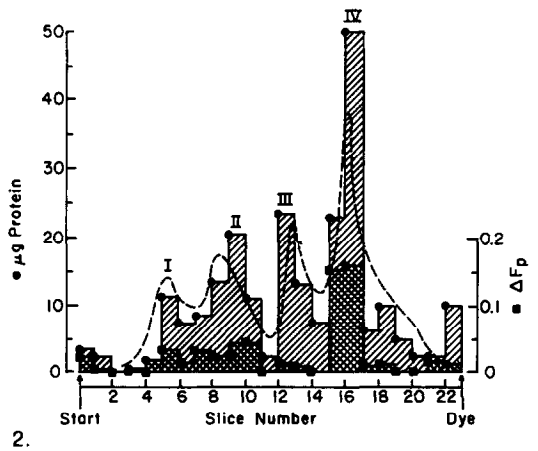


Figure 1: Scatchard plot of the Tb^{3+} binding at 20°C, pH 6.5, to the acetylcholine receptor, the SDS-treated acetylcholine receptor, and the receptor digested with a mixture of trypsin and chymotrypsin. L is the concentration of free Tb^{3+}, and ΔF_p is the observed fluorescence at 546 nm per mg receptor protein. All fluorescence measurements were carried out in 2 mM Pipes buffer containing 0.03% Triton X-100 (pH adjusted with Tris base (Sigma)). •, acetylcholine receptor; $\Delta F_p(max) = 2 \pm 0.04$, $K_D = 25 \pm 1$ μM. o, SDS-treated acetylcholine receptor; after denaturation of the receptor, as described in the text, its concentration was adjusted to 0.05 mg/ml by diluting it with 2 mM Pipes buffer. It was dialysed in Spectrapore #1 dialysis bags for 4 days against a 10-fold volume of Pipes buffer exchanged 9 times; $\Delta F_p(max) = 2.2 \pm 0.2$, $K_D = 24 \pm 1$ μM. □, treated with 2 moles of trypsin and 2 moles of chymotrypsin per mole of acetylcholine receptor in 2 mM Pipes/Tris, 0.03% Triton X-100, pH 6.5, at 4°C, for 48 h. Tb^{3+} fluorescence readings obtained from a solution of the proteases in the buffer, incubated under the same conditions, were used to correct the readings for the treated receptor molecule. This correction amounted to about 10%. 30 μg of treated and untreated receptor were run on analytical SDS-polyacrylamide gels demonstrating that the receptor had been broken down to peptides of molecular weight ≤ 8000; $\Delta F_p(max) = 2.4 \pm 0.2$, $K_{Tb} = 20 \pm 4$ μM.

fore, to be determined by structural features of the peptide chain rather than by the three-dimensional arrangement of the intact receptor molecule. A similar situation exists in prothrombin where the Ca^{2+}-binding sites of the intact protein appear to consist mainly of γ-carboxyglutamic acid residues (30-32). The isolated peptides containing these residues still bind Ca^{2+}, although to a lesser extent than does the protein (32).

The distribution of protein and Tb^{3+} fluorescence in the samples that were obtained after separation of the subunits on an SDS gel is shown in Figure 2. About 35% of the recovered protein is found in the smallest subunit which, as determined on SDS gels (33), has an apparent molecular weight of 40,000. The



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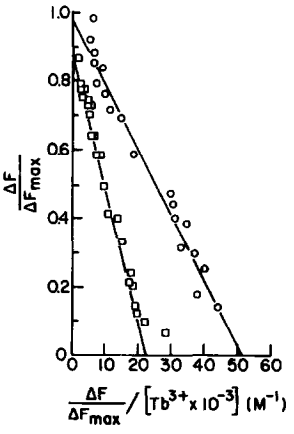

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Figure 2: The isolated acetylcholine receptor subunits, their protein concentration and Tb^{3+}-binding. 600 µg receptor protein were separated on a 6.4% SDS acrylamide gel. Tb^{3+} fluorescence/mg protein, ΔF_p(▩), and protein concentrations (▨) were corrected by subtracting values obtained from a blank gel run in parallel. 350 µg of the original 600 µg protein (58%) was recovered, distributed thus: subunit I, 8%; II, 26%; III, 21%; IV, 35%. Superimposed on the protein profile, as obtained by Lowry analysis of the isolated fractions, is the densitometer trace (---) of a slice that was cut at the side of the gel and stained for protein.
Inset: A Scatchard plot of the binding of Tb^{3+} to the isolated subunit IV in absence (●) and presence (□) of 4.3 mM Ca^{2+}, at 20°C, pH 6.5, 2 mM Pipes-Tris buffer, 0.03% Triton X-100. K_{Tb} = 19 ± 1 µM. K'_{Tb} in presence of 4.3 mM Ca^{2+} = 39 ± 1 µM. Assuming competitive inhibition between Tb^{3+} and Ca^{2+}, $k'_{Tb} = K_{Tb}(1 + [Ca^{2+}]/K_{Ca})$. The value of K_{Ca} obtained from this relationship is 4 ± 0.7 mM.

amount of protein in subunits I-IV is 8, 26, 21, and 35% of the total respectively. The profile of the Tb^{3+} fluorescence per mg protein (▩) shows that the highest specific fluorescence is found with the smallest subunit, IV. In experiments with 9 different receptor preparations, ΔF_p(max) values associated with this subunit were between 1.5 and 2.5 times larger than the values determined for the intact molecule, indicating that most of the Tb^{3+}-binding sites of the receptor are located in subunit IV. The ΔF_p values at 50 µM Tb^{3+} associated with the other subunits were very low and in 2 out of 9 preparations no fluorescence was observed. The inset to Figure 2 shows the binding isotherms of the Tb^{3+}-binding subunit in presence and absence of 4.3 mM Ca^{2+} in the form of a Scatchard plot in which $\Delta F/\Delta F(max)$ gives the fraction of Tb^{3+}-binding sites occupied (16). As can be seen the data are consistent with homogeneous, independent Tb^{3+}-binding

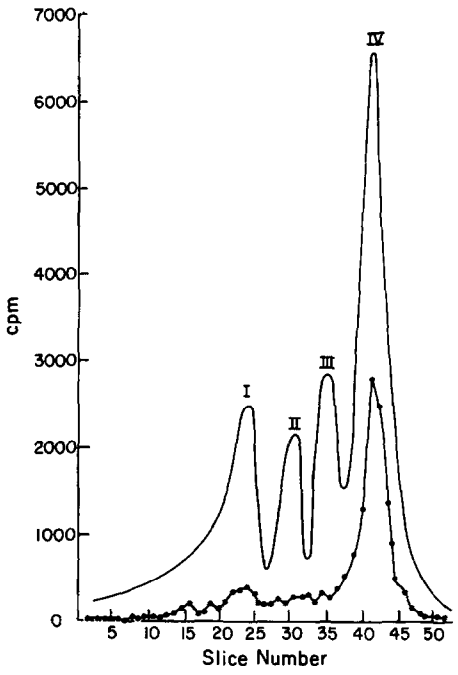

Figure 3: Binding of the affinity-label, [^{3}H]-MBTA, to the receptor subunits. -●-●-, [^{3}H] radioactivity; —, densitometer trace, OD_{560}.
The apparent molecular weights of the subunits, as calculated by comparison with the calibration proteins, phosphorylase A, bovine serum albumin, and ovalbumin, molecular weight = 94,000, 67,000 and 43,000 (33) are: I, 81,000: II, 61,000: III, 50,000 and IV, 40,000.

sites in absence of Ca^{2+} (●). The apparent dissociation constant of these sites, in this experiment, was 19 ± 1 μM. In presence of 4.3 mM Ca^{2+} the apparent dissociation constant for Tb^{3+} increased as is indicated by the increased slope of the line (K'_{Tb} = 39 ± 1 μM). Assuming that Ca^{2+} interacts competitively with the Tb^{3+}-binding sites, the apparent dissociation constant for Ca^{2+} from these sites is 4 ± 0.7 mM, which is about 4 times higher than the value obtained in experiments with the intact receptor. In the native receptor only 60% of the Tb^{3+} sites were found to be affected by 8 mM Ca^{2+} (16).

To determine whether or not the same subunit that carries the Tb^{3+}- and Ca^{2+}-binding sites is also associated with the activator-binding site, the receptor was reacted with [^{3}H]-MBTA before denaturation, then the subunits were separated and the radioactivity determined in each. The distribution of the label among the various subunits is shown in Fig. 3. It can be seen that subunit IV, which

contains most, if not all, the Tb^{3+}-binding sites, also contains most of the affinity label. Attempts to decrease the labeling of the other subunits by either reducing the reaction time or lowering the concentration of the label were unsuccessful, since the overall pattern remained the same and only the total amount incorporated decreased. The amount of label incorporated under the conditions of Figure 3, which do not lead to unspecific labeling of the receptor, was 3 nmoles/mg protein. This value is similar to the value reported by Weill et al. (28), but does not reflect the total number of binding sites measured by α-bungarotoxin.

Our results suggest that it is possible to isolate from hydrolysates of the acetylcholine receptor the peptides which bind Ca^{2+}, and that the use of fluorescent lanthanides may facilitate such studies. Identification of the structural features of the peptides may be an important approach to understanding the role of Ca^{2+} in regulation of permeability changes in neural membranes.

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