CROATICA CHEMICA ACTA CCACAA 47 (3) 425—438 (1975)

CCA-889

YU ISSN 0011-1643
577.15
Conference Paper

Structure and Function of the Acetylcholine Receptor

Mohyee E. Eldefrawi and Amira T. Eldefrawi

Section of Neurobiology and Behavior, Cornell University, Ithaca, N. Y., U.S.A.

The acetylcholine-(ACh-)receptor of electric organs of three *Torpedo* species (*marmorata, ocellata* and *californica*) is purified by affinity chromatography. It acts as a single molecular species as judged by electrophoresis, isoelectrofocusing, gel filtration and immunodiffusion tests. It binds 10 nmoles of ACh or α-bungarotoxin per mg protein. Its amino acids are analyzed and only $\simeq 18\%$ of the cysteic acid residues occur as free SH groups. When the receptor is analyzed for the presence of cations by atomic absorption, 4.7% of its weight is found to be due to bound Ca^{++}. When Ca^{++}-free solutions are used for purification, bound Ca^{++} still contributes 0.6% of its weight. The receptor is an oligomer of an apparent molecular weight of 330 000, with a high tendency to aggregate in the absence of added detergent. It has a doughnut-like appearance with an electron dense core, and consists of 5—6 subunits. Whereas binding of ACh to fresh *Torpedo* membranes has a K_d of 2×10^{-8} M and exhibits positive cooperativity, that to the purified ACh--receptor has similar characteristics, and in addition, has a low affinity component ($K_d = 2 \times 10^{-6}$ M). Antibodies, formed against the ACh-receptor in immunized rabbits or rats, react with the animal's own skeletal neuromuscular ACh-receptors. Incorporation of the ACh-receptor, after mild treatment with trypsin, into oxidized cholesterol bilayers, consistently causes increases in monovalent cation selective conductances (10-over 100 fold), that are triggered by ACh and carbamylcholine and inhibited by curare. The data suggest that the ion conducting carriers or channels of the post-junctional membrane are part of the isolated receptor protein.

In noncellular preparations, the acetylcholine-(ACh-) receptor molecule is identified by its recognition and binding of a variety of reversible ligands, α-neurotoxin or affinity labels. Each group has its advantages and the combination of more than one provides more comprehensive information. α-Bungarotoxin (α-BGT) is the most popular because of its very high affinity and specificity for, as well as the relative irreversibility of its binding to the nicotinic ACh-receptors. However, some treatments that alter the receptor and do not affect toxin binding, modify the binding of the transmitter ACh. For example, the purified receptor of *T. marmorata* eluted off the affinity gel with 100 mM benzoquinonium binds [^{125}I]α-BGT, but not [acetyl-^{3}H]acetylcholine[1]. Also a single freezing and thawing of a Triton extract of *T. californica* totally destroys its ACh-binding, but reduces its ability to bind α-BGT by only 30%[2].

There are now available in different laboratories several purified ACh--receptors from a variety of electric organs (Table I). Three observations are

of interest: one is the ratio of $\simeq 2$ for the maximum α-BGT or cobra toxin binding to maximum ACh-binding per mg protein occasionally observed[3-5]. We find equal maximum binding of the two ligands of 10 nmol/mg protein for the receptors purified from three fresh *Torpedo* species (*marmorata* (unpublished), *ocellata*[2] and *californica*[6]). This difference in maximum binding of the two ligands may be due to alterations that occur in the receptor molecule during its purification, which affect only the ACh-binding. The second observation is that protein concentration determined by amino acid analysis is usually lower than that determined by the Lowry *et al.* method. Thus specific binding based on the first is higher by 10—30% than that based on the second[1,7,8]. The last is the large difference in Triton concentration in the various preparations, which may account for many of the discrepancies in the properties of purified receptors.

CHARACTERISTICS OF ACh-BINDING OF THE PURE RECEPTORS

Binding of [acetyl-^{3}H]acetylcholine to purified receptors from three *Torpedo* species (*marmorata, ocellata* and *californica*) indicate the presence of a high affinity component ($K_d = 2 \times 10^{-8}$ M) and a low affinity one ($K_d = 2 \times 10^{-6}$ M). Binding of [^{3}H]ACh at very low concentrations ($< 10^{-8}$ M) indicates positive cooperativity (Hill coefficient $1.5 \pm .2$) (Fig. 1)[9]. If we compare this with the binding of the receptor from the three *Torpedo* species, when still in fresh membranes or solubilized in 1% Triton X-100, we find only the high affinity component ($K_d = 8 \times 10^{-9} - 2 \times 10^{-8}$ M)[2,8], which is also highly cooperative (Fig. 1). The Hill coefficient ranges from 1.5—1.8[2]. In 1971, we reported on

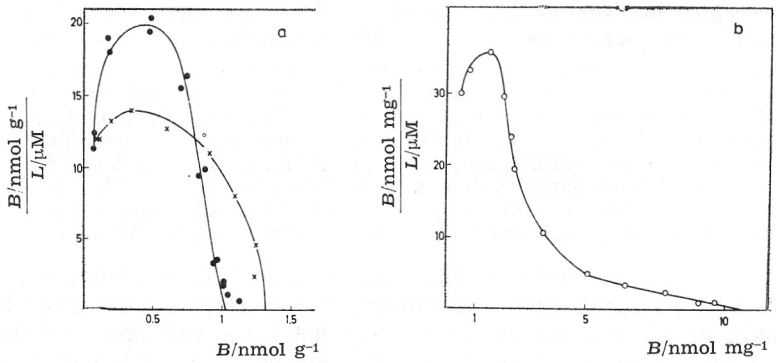

Fig. 1. Scatchard plot of the binding of [acetyl-^{3}H]acetylcholine to the *T. ocellata* ACh-receptor, measured by equilibrium dialysis at 4 °C for 16 hr. (a)-Binding to crude receptor preparations, membrane-bound x——x; 1% Triton X-100 solubilized ●——●. (b)-Binding to the purified receptor. B, nmoles bound per g tissue in (a) and per mg protein in (b). L, concentration of [acetyl-^{3}H]acetylcholine in μM.

the presence of both high and low affinity ACh-binding by the lyophilized membranes of *T. marmorata*, which were stored at — 20 °C for 3 years[10] The lower affinity for ACh may be accompanied by an increase in affinity for α-BGT[11]. The change in affinity for ACh by the membrane bound receptor is apparently due to some modification in the receptor. It has been shown that a variety of treatments, such as incubation at 40 °C for 40 min or treatment with 10^{-4} M PCMB or DTNB cause lowering of the affinity of the Triton-

-solubilized ACh-receptor for ACh[12]. On the other hand similar heat treatment of *T. ocellata* electroplax membranes had no effect on its affinity for ACh, except in presence of 1% Triton X-100. Insulin receptors on cultured lymphocytes and liver plasma membranes have been shown to exhibit negative cooperativity in their binding of insulin[13].

The question of whether or not the low affinity ACh-binding that appears in the purified ACh-receptor from the three *Torpedo* species and *E. electricus* (unpublished) is related to the low affinity ACh-binding, that appears in the aged *T. marmorata,* is still unanswered. It is possible that the low affinity ACh--binding is a result of the activity of endogenous esterases, proteases, oxidation or reduction that may take place during purification. However, the presence of 10^{-5} M DFP, as an esterase and protease inhibitor, during purification, does not cancel this low affinity component. Another possibility is that the removal of the receptor molecule from its lipid or Triton environments changes the nature of the subunit interactions, possibly leading to negative cooperativity.

This observation of a change in the affinity of some of the ACh-binding sites after receptor purification, led us to investigate whether other properties of the molecule have also changed. An interesting finding is that although the presence of 1% Triton X-100 does not affect the high affinity of ACh-binding by the crude receptor, that of the pure receptor becomes very sensitive to Triton X-100; reducing its high affinity without affecting the maximum number of ACh-binding sites (Fig. 2). Another is the sensitivity of the pure ACh-

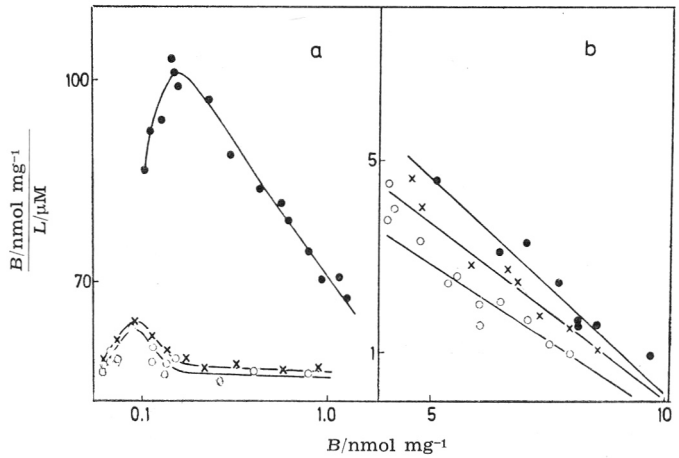

Fig. 2. Scatchard plot of the data showing the effect of Triton X-100 (added to bath and sac contents) on the binding of [acetyl-^{3}H]acetylcholine to the purified *californica* ACh-receptor, determined by equilibrium dialysis at 4 °C for 16 hr. Control ●——●; x——x, or 1% Triton X-100 ○——○. B, amount bound in nmoles/mg protein; L, concentration of [acetyl-^{3}H]acetylcholine in μM. (a)-Binding at low ACh concentrations ($8 \times 10^{-10} - 10^{-8}$ M) (b)-Binding at high ACh concentrations (10^{-6} M — 8×10^{-6} M) (from Edelstein *et al.*[6]).

-receptor to freezing and thawing which reduce its ACh-binding at 10^{-6} M by 30%, without affecting the membrane bound receptor.

STRUCTURE AND COMPOSITION

Because of the low amount of Triton X-100 present in our *marmorata* purified receptor, rough estimate of the maximum molecular weight was obta-

ined by sedimentation velocity studies in a Beckman Model E ultracentrifuge equipped with a scanner and an on-line computer system[14]. Later, we determined the exact amount of Triton X-100 in the purified receptor, using [^{3}H]Triton X-100, and found it to be 0.113 mg/mg protein[6]. The molecular weights of three purified receptor preparations were then determined by sedimentation equilibrium[6]. Two molecular weight species are found in each case with a common one of apparent molecular weight of 330 000. In addition, the second species of the *californica* purified receptor has an apparent molecular weight of 660,000 and that of *marmorata* (Fig. 3) or *E. electricus* is

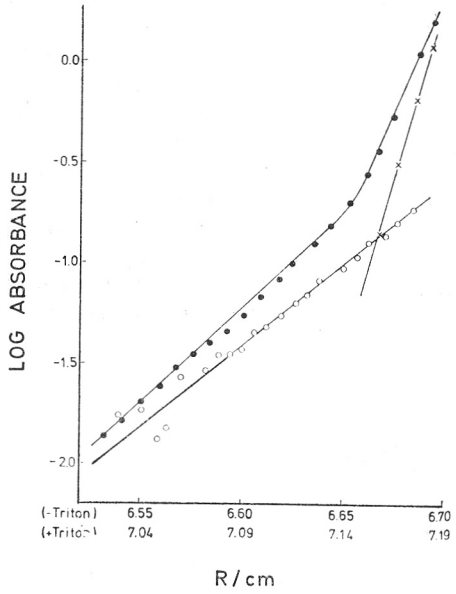

Fig. 3. Sedimentation equilibrium results on the purified *marmorata* ACh-receptor. Data is presented as log absorbance versus R rather than R^2 since R is effectively proportional to R^2 over the limited range of values encountered. Solutions with no added Triton X-100 ●——●, with 0.2% Triton X-100 ○——○. Data for the heavier species in the absence of added Triton X-100 x——x is obtained by subtracting extrapolated values of the concentration of the lighter species from the concentrations at corresponding radial positions beyond the break in the curve (from Edelstein *et al.*[6]).

1 300 000. However, in presence of Triton X-100 (above 0.1%), only the smaller species of 330 000 is observed (Fig. 4). This raises the question as to whether the native ACh-receptor in the membrane is the 330 000 unit or higher aggregates. Estimates of the molecular weight of the receptor protomer, based on ligand binding, depend on the purity of the preparation and denaturation, if any, that may occur during purification. The smallest unit calculated from specific binding of the purest preparations ranges from about 80 000 to 100 000[1,3,4]. If we consider 330 000 as the molecular weight of the native receptor oligomer and the protomer is 80 000, we may conclude that the receptor molecule is a tetramer. On the other hand, if the receptor is 660 000 and the protomer is about 100 000; the receptor in the membrane may be a hexamer that is split in half by Triton, or a trimer that aggregates upon removal from its lipid or Triton environment.

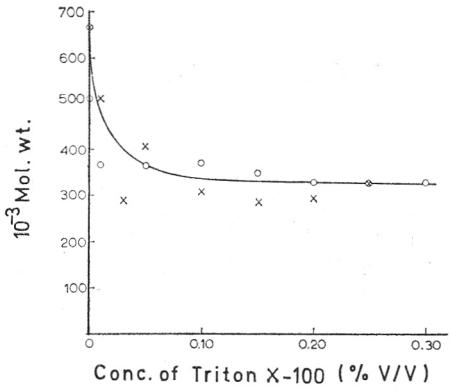

Fig. 4. Dependence of apparent molecular weight on concentration of Triton X-100 for receptor from *T. californica* O——O and *T. marmorata* x——x (from Edelstein *et al.*[6]).

The subunits of the ACh-receptor, obtained by SDS gel electrophoresis, vary from a single subunit[15] to several, with most detecting two subunits[3,16,17]. This may depend on the purity of the receptor and the extent of the denaturing treatment used prior to electrophoresis. We obtain a major subunit of 43 000[2] and two minor ones of larger molecular weight. The larger bands appearing in the gel are apparently receptor molecules partially dissociated by the SDS and mercaptoethanol treatment. It is suggested that the ACh-receptor molecule is composed of unidentical subunits. Binding and kinetic data on the ACh-receptor have led to suggestions that activators and antagonists bind to different sites[18,19]. If this is so, it would be interesting to determine whether these different binding sites are on the same or different subunits.

Electron micrographs of the negatively stained pure ACh-receptor show it as a doughnut with an electron dense core and a subunit structure[2,20]. This is similar to the hexagonal structures observed in the synaptic membranes of electric organs[20]. An interesting aspect is that similar hexagonal arrays were observed in the luminal membrane of urinary bladder, as well as in 'gap junctions' of liver[21] and goldfish brain[22], where no ACh-receptors are known to exist. It is plausible that this array is important for regulation of ion fluxes in special membranes, and in the case of cholinergic postsynaptic membranes the molecules are modified by the addition of ACh-binding sites, thus becoming ACh-receptors.

The ACh-receptor is a protein possibly containing hexosamines[3,4,23]. The amino acid composition of our purified *californica*[2] and *marmorata*[1,2] receptors was analyzed by Dr. D. Wilson (Table II). The receptor contains 21.6—22.5 mol % acidic amino acids and 11.7 mol % basic ones, which is reflected in its acidic nature (pI of 4.6—4.8)[1,2]. Its free SH content differs according to the conditions to which it is exposed. Maximum number of free SH groups we obtain in the *californica* receptor is 20 nmol/mg protein (equal to 18% of the total cysteic acid residues), if its source is a fresh electric organ, deaerated solutions are used for its purification and its final dialysis is performed under nitrogen[8]. By contrast, the ACh-receptor prepared from *marmorata* electric organ, that is lyophilized and stored at —20 °C for 5 years, contains 2.4 nmol

TABLE I.

Summary of the affinity chromatography methods used for purification of ACh-receptors from the electric organs of three fish species and certain properties of these receptors. The first seven receptors are from E. electricus, the next two from T. marmorata, the following three from T. californica and the last one from T. nobiliana.

Immobilized ligand	Ligand (in mM) for desorption of receptor	Specific binding[a]		ACh-receptor/ACh-esterase	% Detergent in purified receptor solution	Ref.
		α-toxin	ACh			
Flaxedil analog	flaxedil-1	2—3.3	—	100	1% Triton X-100	30
Flaxedil analog	flaxedil-2.5	5.5—6.5	2.2—2.6[b]	100	1% Triton X-100	4
α-Toxin, *Naja naja*	hexamethonium-50	6.6	—	2000	0.5% Tween 80	15
Phenyltrimethylammonium	carbamylcholine-50	2—4[c]	—	50	0.2% Triton X-100	31
Phenyltrimethylammonium	decamethonium-1	4.5	—	140	1.5% Triton X-100	32
α-Toxin, *Naja naja*	decamethonium-10	4.1	—	—	0.5% Triton X-100	17
Phenyltrimethylammonium	Bis-Q[d]-0.003	5—6.5	2.5—3.3	2000—13,000	0.01% Brij	5
α-Toxin, *Naja naja*	carbamylcholine-500	2.3	—	—	1% Triton X-100	33
α-Toxin, *Naja naja*	carbamylcholine-1000	—	7.8[a], 10.4[e]	20,000	0.0007% Triton X-100	1
Quaternary ammonium	NaCl-500	6	3[f]	—	0.1% Triton X-100	34
Quaternary ammonium	NaCl-500	10	5[f]	—	—[g]	3
α-Toxin, *Naja naja*	carbamylcholine-1000	10	10	20,000	0.0007% Triton X-100	8
Phenyltrimethylammonium	carbamylcholine-50	6		2,000	0.2% Triton X-100	35
α-Toxin, *Naja naja*	carbamylcholine-100	12.2[e]		20,000	0.1% Triton X-100	36

[a]Expressed in nmol/mg protein, which is based on analysis by Lowry *et al.* method. [b]Binding in presence of 1% Triton X-100. [c]Binding of 4-(*N*-maleimido)-benzyltrimethylammonium iodide. [d]3,3′-bis[α-(trimethylammonium)methyl]-azobenzene bromide. [e]Based on protein value obtained by amino acid analysis. [f]Binding of ACh in the presence of 0.1% Triton. [g]For reconstitution experiments, concentration of Triton was 0.0105 mg/mg protein.

free SH/mg protein. The low SH content is suggested to be the reason for its lower specific binding of ACh of 7.8 nmol/mg protein[8].

Because of the low Triton content of our purified receptor, we are able to detect the native fluorescence of the molecule[8]. Maximum u. v. absorption of he receptor is at 290 nm, and when excited, its maximum emission is at 36 nm (Fig. 5). Thus, as with most proteins[24], this fluorescence due to L-try-

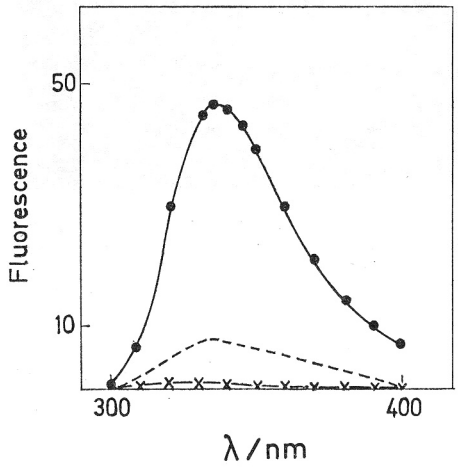

Fig. 5. Fluorescence emission spectra of the purified *californica* or *marmorata* ACh-receptors (0.1 mg/ml), excited at 290 nm ●——●, Triton X-100 (0.0007%) x——x, and bovine serum albumin (0.1 mg/ml) containing 0.0007% Triton X-100 - - - - - - (from Eldefrawi *et al.*[8]).

ptophan in the molecule is shifted to shorter wavelengths by 12 nm than the free L-tryptophan. A concentration of receptor as low as 40 ng/ml is easily detectable by its fluorescence. Unfortunately, this fluorescence is unchanged by addition of 10^{-7} — 10^{-5} M ACh, decamethonium, carbamylcholine or *d*-tubocurarine[8]. However, the fluorescent cations Eu^{+++} and Tb^{+++} bind to the purified ACh-receptor and this binding is accompanied by a large enhancement of their fluorescent emission. A solution of 2×10^{-4} M Eu^{+++} or 5×10^{-5} M Tb^{+++}, excited at 398 nm or 295 nm, show weak emission maxima at 620 nm and 546 nm, respectively. Addition of ACh-receptor increases the emission intensity by a factor of 20 for Eu^{+++} and 70 for Tb^{+++}. Studies of the effects of ions and activators on the fluorescent receptor-ion complexes are in progress[25].

IMMUNOLOGICAL CHARACTERIZATION OF THE ISOLATED ACh-RECEPTOR

In collaboration with Dr. N. Norcross, we found that injection of a very small dosage of the purified *californica* receptor intramuscularly into rabbits (25 μg) or rats (12 μg) is effective in stimulating antibody production[26]. Reaction to immunization in rabbits is severe and rapid, for three weeks after inoculation 7 out of 8 immunized rabbits became paralyzed and then died. In immunodiffusion tests, a sharp and strong immunoprecipitin line forms between pure antigen and antisera[2,26], but multiple or thick lines form between crude receptor and its antisera (Fig. 6). Rabbit antisera precipitate the crude or pure ACh-receptor totally, but the receptor antibody precipitate retains some capacity to bind ACh (Fig. 7). However, if the concentrated γ-globulin fraction

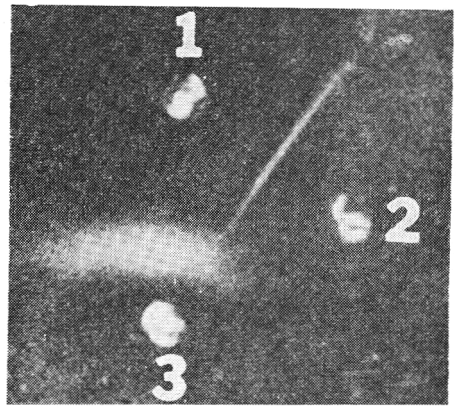

Fig. 6. Immunodiffusion assay of pure (Well 2) and crude (Well 3) *californica* ACh-receptor preparations against antiserum from rabbit immunized with crude ACh-receptor (Well 1) (from Norcross and Eldefrawi[26]).

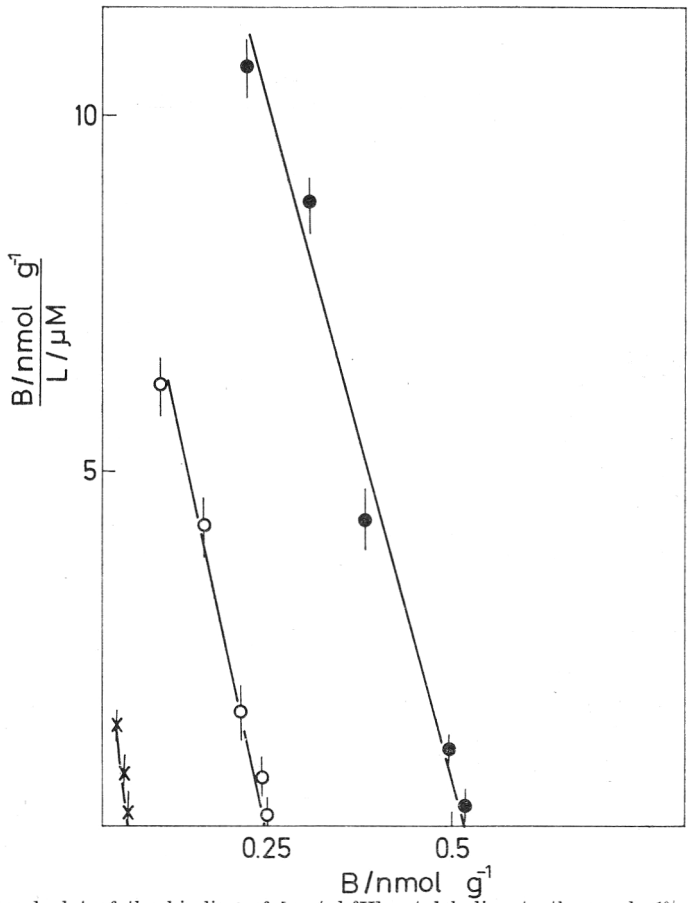

Fig. 7. Scatchard plot of the binding of [acetyl-^{3}H]acetylcholine to the crude 1% Triton extract of the *californica* ACh-receptor control ●——●. Binding to the precipitate formed after overnight incubation at 4 °C with an equal volume of rabbit antisera ○——○; binding to the unprecipitated receptor x——x. B, nmol bound per g tissue; L, concentration of free [acetyl-^{3}H]acetylcholine in μM.

is incubated with the receptor, there is total inhibition of ACh-binding in the precipitate.

When the evoked potential-EMG of the muscles of the rabbits, immunized with our *californica* receptor, was measured by Sanders *et al.*[27], greater than 50% decrement in amplitude was shown at stimulation frequencies at or less than 10 per second. The decrement was partially reversed by injection of neostigmine or edrophonium (Fig. 8).

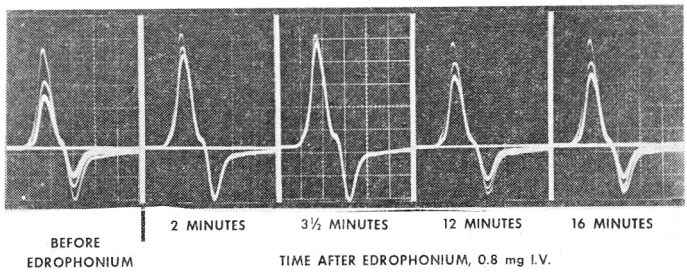

Fig. 8. Evoked potential EMG of the gastrocnemius muscle of a rabbit immunized with the purified *californica* ACh-receptor; superimposed potentials, at 10/s stimulation before and after intravenous injection of 0.8 mg edrophonium. The decrement almost disappears by 3½ min, but reappears as the effect of edrophonium wears off (from Sanders *et al.*[27]).

Contrary to rabbits, the general appearance of inoculated rats is normal for 4 weeks, although they develop as high a titer of antibodies as the fatally paralyzed rabbits. The defect in neuromuscular transmission is transient, appearing 8 to 14 days after inoculation and lasting up to 3 days[27]. There is also a progressive reduction in the mean corrected MEPP amplitude of diaphragm preparations *in vitro* beginning after 10 days. It is suggested that injection with the ACh receptor stimulates the development of cellular and/or humoral immunological response against the nicotinic receptor of skeletal muscles.

FUNCTION OF THE ACh-RECEPTOR

Binding of ACh to its receptor in the postsynaptic membrane of excitatory junctions cause specific cation fluxes across that membrane. Therefore, the first function of this receptor is to recognize and bind ACh, and this we have shown for the purified ACh-receptors. The second function is to trigger increases in monovalent cation conductance of the cell membrane of which it is a part, by acting, as a whole or in part, as a carrier or channel, or by affecting a separate translocation machinery. The ACh-receptor we purified from *T. californica* acts as a single molecular species in disc gel electrophoresis, isoelectrofocusing, gel filtration and in immunodiffusion tests[2,8,26]. Therefore, if it can increase specific cation conductance of bilayers in response to activators, that would strongly suggest that part or all of the receptor molecules is acting as a selective cation carrier or channel.

When the purified receptor is added to 5 ml of the desired electrolyte solution and 5 mM histidine (pH=7.3) in each of two compartments separated by 1 mm surface area, and a bilayer of oxidized cholesterol is then formed increases in bilayer conductance are observed in the presence of 10^{-1} M NaCl[2]. Their magnitude and rate increase as the concentration of receptor is raised

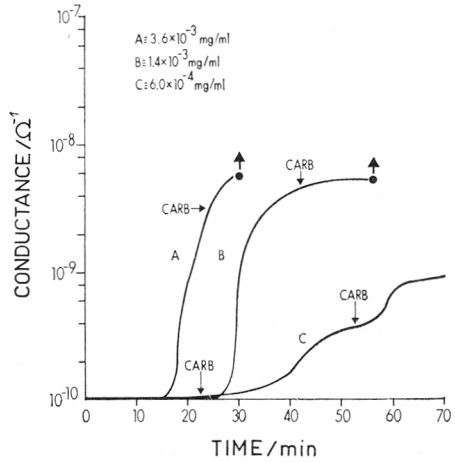

Fig. 9. The time response of bilayer conductance at 23 °C in the presence of 0.1 M NaCl and the purified *californica* ACh-receptor. The arrow next to carb indicates addition of 10 μM final concentration of carbamylcholine. The ACh-receptor is used at the three concentrations indicated for A, B and C. Data are representative of five experiments. Conductance of bilayer is continuously monitored under the condition of triangular waveform of ± 75 mV maximum voltage clamp at a frequency of 0.015 Hz (from Eldefrawi *et al.*[2]).

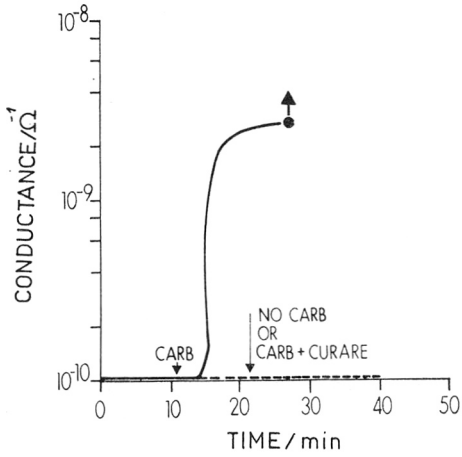

Fig. 10. The increase in bilayer conductance in presence of 0.1 M NaCl and the purified trypsin-treated *californica* ACh-receptor (0.8 μg/ml), resulting from the addition of carbamylcholine at a final concentration of 10 μM. Concentration of curare is 10 μM. Data are representative of five experiments (from Eldefrawi *et al.*[2]).

(Fig. 9). However, addition of ACh or carbamylcholine sometimes increases Na^+ conductance as in line C, and sometimes not. However, if the receptor is first incubated with trypsin (50 : 1 w/w) for 1 hr at 23 °C, then soybean trypsin inhibitor added, and positively charged peptides removed by ion exchange chromatography on Sephadex CM50, monovalent cation selective conductances are observed. In the presence of 10^{-1} M Na^+, the addition of carbamylcholine or ACh (10^{-5} M) to a steady state causes a dramatic increase in conductance (Fig. 10). This increase in Na^+ conductance is observed in every single experiment, but its magnitude varies from 10 to over 100 fold. If curare is present, then carbamylcholine is added after the formation of the bilayer and no

TABLE II.

Amino acid composition (mol %) *of ACh-receptors purified from electric organs of two* Torpedo *sp.*

Amino acid	*marmorata*	*californica*
Lysine	6.1	5.4
Histidine	2.1	2.4
Arginine	3.5	3.9
Aspartic acid	11.8	11.6
Threonine	6.3	6.4
Serine	7.1	7.9
Glutamic acid	10.7	10.0
Proline	6.2	5.9
Glycine	6.4	4.6
Alanine	6.0	5.0
Cysteic acid	2.0	1.2
Valine	5.5	7.1
Methionine	1.7	2.0
Isoleucine	5.2	8.2
Leucine	9.3	9.5
Tyrosine	3.6	3.7
Phenylalanine	4.4	4.5
Tryptophan	2.1	2.4
% Polar residues[a]	48	47

[a]Sum of Asp, Glu, Lys, Ser, Arg, Thu and His.

increase in conductance is observed. No selectivity difference is found between Na^+ and K^+. However, when diffusion potentials are measured, sodium permeability is 4.4 fold that of Cl^- permeability.

It appears that what the trypsin treatment does is to permit the incorporation of the receptor in bilayer in a functional conformation through newly exposed side chains. This is because the receptor's binding of ACh is unchanged and so is its mobility in 7% polyacrylamide gel electrophoresis. However, the size of its subunits is reduced (Fig. 11), but they remain attached possibly through disulfide, hydrogen and hydrophobic bonds.

An observation that is at first confusing is the finding that bilayer conductance is increased in the presence of the receptor or trypsin-treated receptor, when Ca^{++} is the cations present in the bathing fluid (Fig. 12). This conductance is dependent on the concentrations of cation and receptor, but not sensitive to the addition of carbamylcholine or ACh, and there is no selectivity between Ca^{++} and Cl^-. Similar results were obtained with a mitochondrial glycoprotein, which has a high affinity for Ca^{++} and causes non-selective increase in bilayer conductance[28]. It is possible that these non-selective conductances may be due to a chemical event, the interaction of Ca^{++} receptor with lipids. We find that the receptor binds Ca^{++} with two affinities, one is low, calculated through its inhibition of ACh ($K_i = 7 \times 10^{-3}$ M), and the other is very high and detected by atomic absorption[29]. In fact, when purified in presence of 0.67 mM Ca^{++},

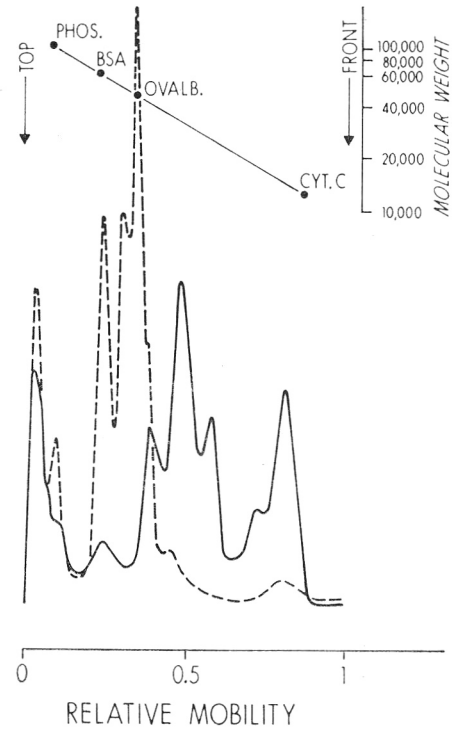

Fig. 11. Scan of SDS-gel electrophoresis of the purified *californica* ACh-receptor. Straight line, receptor after incubation with trypsin (50 : 1 w/w) for 1 hr; broken line-untreated receptor. Standards are run on a separate gel. Phos, phosphorylase (94 000); BSA, bovine serum albumin (66 000); Oval, ovalbumin (43 000); Cyt, c, cytochrome c (11 700) (from Eldefrawi *et al.*[2]).

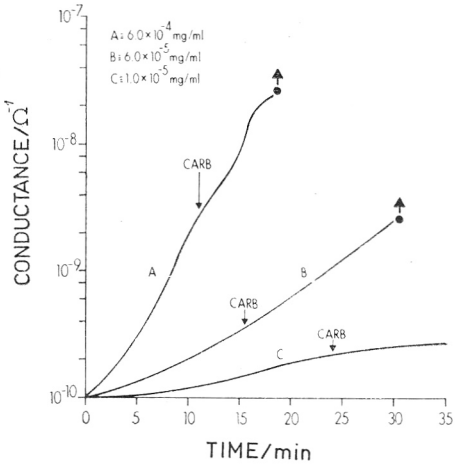

Fig. 12. The time response of bilayer conductance in the presence of 3 mM $CaCl_2$, 5 mM histidine, pH = 7.3. The trypsin treated *californica* ACh-receptor is used at the indicated concentrations for A, B and C. Final concentration of carbamylcholine is 10 μM. Data are representative of six experiments (from Eldefrawi *et al.*[2]).

4.7% of the weight of the ACh-receptor is due to Ca^{++}. Even when Ca^{++} free solutions (containing 1 mM EDTA) are used during purification, 0.6% of the molecular weight is still due to bound Ca^{++}, equivalent to 15 moles of Ca^{++} per mole of ACh bound at saturation.

The steady state increased monovalent cation conductance of the bilayer, caused by the trypsin-treated ACh-receptor, its cation selectivitiy, its dependence on activators and inhibition with curare are consistent with the *in vivo* function of the receptor. However, the magnitude of the increase is apparently much below that occurring *in vivo,* where there is a chemical gradient and the membrane lipid is definitely not oxidized cholesterol.

In summary we may claim that we have learned some of the structural properties of the ACh-receptor protein but we realize that a major effort is still needed to learn the exact nature and the arrangement of its subunits. Similarly, while our data, as well as others, on the effect of the receptor on bilayer conductances suggest that the regulatory mechanism of ion translocation is part of the isolated receptor molecule, these effects are far too small in magnitude when compared to the physiological event. In short, we can only claim that, while we may see the light at the end of the tunnel, we still have a long enjoyable journey ahead of us.

Acknowledgment. This work was supported in part by research grant NS-09144 from the National Institutes of Health, U.S.A.

REFERENCES

1. M. E. Eldefrawi and A. T. Eldefrawi, *Arch. Biochem. Biophys.* **159** (1973) 362.
2. M. E. Eldefrawi, A. T. Eldefrawi, and A. E. Shamoo, *Annals N.Y. Acad. Sci.,* in press.
3. D. Michelson, R. Vandlen, J. Bode, T. Body, J. Schmidt, and M. A. Raftery, *Arch. Biochem. Biophys.* **165** (1974) 796.
4. J.-C. Meunier, R. Sealock, R. Olsen, and J.-P. Changeux, *Eur. J. Biochem.* **45** (1974) 371.
5. H. W. Chang, *Proc. Nat. Acad. Sci. U.S.A.* **71** (1974) 2113.
6. S. J. Edelstein, W. B. Beyer, A. T. Eldefrawi, and M. E. Eldefrawi, *J. Biol. Chem.,* in press.
7. J. Schmidt and M. A. Raftery, *Biochemistry* **12** (1973) 852.
8. M. E. Eldefrawi, A. T Eldefrawi, and D. B. Wilson, *Arch. Biochem. Biophys.,* in press.
9. M. E. Eldefrawi and A. T. Eldefrawi, *Biochem. Pharmacol.* **22** (1973) 3145.
10. M. E. Eldefrawi, A. G. Britten, and A. T. Eldefrawi, *Science* **173** (1971) 338.
11. R. D. O'Brien and R. E. Gibson, *Arch. Biochem. Biophys.* **165** (1974) 681.
12. R. D. O'Brien and R. E. Gibson, *Arch. Biochem. Biophys.,* in press.
13. P. De Meyts, J. Roth, D. M. Neville, Jr., J. R. Gavin III, and M. A. Lesnik, *Biochem. Biophys. Res. Commun.* **55** (1973) 154.
14. R. H. Crepeau, C. P. Hensley, and S. J. Edelstein, *Biochemistry* **13** (1974) 4860.
15. R. P. Klett, B. W. Fulpius, D. Cooper, M. Smith, E. Reich, and L. D. Possani, *J. Biol. Chem.* **248** (1973) 6841.
16. F. Hucho and J.-P. Changeux, *FEBS Lett.* **38** (1973) 11.
17. J. Lindstrom and J. Patrick, in: M. V. L. Bennett (Ed.), *Synaptic Transmission and Neuronal Interaction,* Raven Press, New York 1974, pp. 191—216.
18. M. E. Eldefrawi, in: J. I. Hubbard (Ed.), *The Peripheral Nervous System,* Plenum Press, New York 1974, pp. 181—200.

19. J. L. Fu, D. B. Donner, and G. P. Hess, *Biochem. Biophys. Res. Commun.* **60** (1974) 1072.
20. J. Cartaud, E. L. Benedetti, J. B. Cohen, J.-C. Meunier, and J.-P. Changeux, *FEBS Lett.* **33** (1973) 109.
21. E. L. Benedetti and P. Emmelot, *J. Cell. Biol.* **38** (1968) 15.
22. G. Zampighi and J. D. Robertson, *J. Cell. Biol.* **56** (1973) 92.
23. W. M. Moore, L. A. Holladay, D. Puett, and R. N. Brady, *FEBS Lett.* **45** (1974) 145.
24. L. Brand and B. Withholt, *Methods Enzymol.* **11** (1967) 776.
25. H. Rübsamen, A. T. Eldefrawi, M. E. Eldefrawi, and G. P. Hess, *Fed. Proc.* **34** (1975) 594.
26. N. L. Norcross and M. E. Eldefrawi, *J. Immunol.* in press.
27. D. B. Sanders, L. S. Schleifer, E. E. Cobb, N. L. Norcross, and M. E. Eldefrawi, to be presented at *Int. Conf. on Myasthenia Gravis, Annals N.Y. Acad. Sci.*, in prep.
28. G. Prestipino, D. Ceccarelli, F. Conti, and E. Carafoli, *FEBS Lett.* **45** (1974) 99.
29. M. E. Eldefrawi, A. T. Eldefrawi, L. A. Penfield, R. D. O'Brien, and D. Van Campen, *Life Sci.*, in press.
30. R. W. Olsen, J.-C. Meunier, and J.-P. Changeux, *FEBS Lett.* **28** (1972) 96.
31. A. Karlin and D. Cowburn, *Proc. Nat. Acad. Sci. U.S.A.* **70** (1973) 3636.
32. G. Biesecker, *Biochemistry* **12** (1973) 4403.
33. E. Karlsson, E. Heilbronn, and L. Widlung, *FEBS Lett.* **21** (1972) 107.
34. J. Schmidt and M. A. Raftery, *Biochem. Biophys. Res. Commun.* **49** (1972) 572.
35. C. L. Weill, M. G. McNamee, and A. Karlin, *Biochem. Biophys. Res. Commun.* **61** (1974) 997.
36. D. E. Ong and R. N. Brady, *Biochemistry* **13** (1974) 2822.

SAŽETAK

Struktura i funkcija receptora acetilkolina

Mohyee E. Eldefrawi i Amira T. Eldefrawi

Receptor acetilkolina (ACh-receptor) električnog organa iz tri različite vrste *Torpedo (marmorata, ocellata* i *californica)* čišćen je metodom afinitetne kromatografije. U toku elektroforeze, isoelektričnog fokusiranja, gel-filtracije i imunodifuzijskih testova, pročišćeni ACh-receptor ponaša se poput jedne molekularne vrste. Receptor veže po miligramu proteina 10 nmol ACh ili α-bungarotoksina. Analiza aminokiselina pokazala je da samo 18% ostataka cisteinske kiseline ima slobodne SH-grupe. Analizom receptora na prisutnost kationa metodom atomske apsorpcijske spektrofotometrije nađeno je da 4.7% težine otpada na vezani Ca^{2+}. Istovrsna analiza nakon čišćenja receptora iz otopina bez Ca^{2+}, pokazala je da na vezani Ca^{2+} otpada još uvijek 0.6% težine. ACh-receptor je oligomer s prividnom molekularnom težinom od 330 000 i u mediju bez detergenta pokazuje veliku sklonost agregaciji. Kolutastog je oblika s gustom elektronskom jezgrom i sastoji se od 5—6 podjedinica. Dok vezanje ACh na svježe membrane *Torpeda* ima $K_d = 2 \cdot 10^{-8}$ M i pokazuje pozitivnu kooperativnost, vezanje na pročišćeni receptor ima slične karakteristike no vrlo niski afinitet ($K_d = 2 \cdot 10^{-6}$ M). Antitijela formirana na ACh-receptor u imuniziranim kunićima ili štakorima, reagiraju sa skeletnim neuromuskularnim ACh-receptorima iste životinje. Ugrađivanje ACh-receptora, nakon blage obradbe tripsinom, u oksidirane dvoslojeve kolesterola, dosljedno uzrokuje porast selektivnih vodljivosti monovalentnih kationa (od 10 do preko 100 puta), koje su inicirane s ACh i karbamilkolinom, a inhibirane s kurare. Rezultati ukazuju da su nosači iona ili kanali postsinaptičke membrane sastavni dijelovi izoliranog receptorskog proteina.

SEKCIJA ZA NEUROBIOLOGIJU I PONAŠANJE
SVEUČILIŠTE CORNELL,
ITHACA, N. Y, U. S. A.