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Endogenous and Exogenous Proteolysis of the Acetylcholine Receptor From Torpedo californica

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Purified acetylcholine receptor reconstituted into liposomes catalyzes carbamylcholine-dependent ion flux [10]. An endogenous protease activated by Ca^{2+} gives rise to an acrylamide gel pattern of the receptor with the 40,000-dalton subunit apparently as the major component. Exogenous proteases nick the proteins so extensively that the acrylamide gel pattern reveals polypeptides of 20,000 daltons or less. In either case the receptor sediments at 9S, indicating that the polypeptide chains remain associated. Moreover, the nicked receptors bind α-bungarotoxin and catalyze carbamylcholine-dependent ion flux after reconstitution.

Key words: properties of acetylcholine receptor, reconstitution of acetylcholine receptor, subunit composition of acetylcholine receptor, proteolysis of acetylcholine receptor, Ca^{2+} activated protease

The nicotinic acetylcholine receptor plays an important role in the synaptic transmission of nerve impulses. Binding of acetylcholine to the receptor triggers a transient opening of channels in the postsynaptic membrane, which allows Na^+ and K^+ ions to diffuse down their electrical chemical gradients [1, 2, 3].

The acetylcholine receptor has been purified from many sources, including the electrical organs of Electrophorus electricus, Torpedos [4, 5] and also from skeletal muscle [6]. The subunit composition of the purified receptor remains controversial. Receptor purified from detergent extracts of Torpedo membranes by affinity chromatography usually show four subunits of molecular weights 40,000 (α), 48,000 (β), 58,000 (γ) and 64,000 (δ) with a stoichiometry of $\alpha_2\beta\gamma\delta$ for the monomer of 250,000 molecular weight [7]. The α subunit binds acetylcholine and affinity analogs, as well as α-bungarotoxin [1]. The functions of the other subunits are not known, but may be involved in channel formation [3].

Recently it has been reported that a membrane preparation consisting mainly of the 40,000 molecular weight subunit is active in ion translocation, suggesting that the other peptides are contaminants or not involved in ion translocation [8, 9]. We have recently reported that a purified acetylcholine receptor which contains only the α, β,

Abbreviations: SDS – sodium dodecyl sulfate; PMSF – phenylmethylsulfonylfluoride; [^{3}H]-BTX – N-[propionyl-^{3}H] α-bungarotoxin.

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and δ subunits can be reconstituted into liposomes which catalyze a carbamylcholine-dependent ion flux [10]. In this communication we show that precautions must be taken during the preparation of the acetylcholine receptor because it is very susceptible to proteolysis by endogenous Ca^{2+}-activated [1] and NEM-sensitive [11] protease which cleave the γ as well as the β and δ subunits without affecting the properties of the receptor. We also show that pronase can nick all the subunits of the receptor so that the SDS gel patterns are drastically changed with none of the original subunits remaining intact. Yet a receptor nicked in this manner sediments as a 250,000-dalton complex, binds α-bungarotoxin, and catalyzes a carbamylcholine-sensitive ion flux after reconstitution.

MATERIALS AND METHODS

Purification and Reconstitution of Acetylcholine Receptor

Acetylcholine receptor-rich membranes were prepared from the electric organs of Torpedo californica (Pacific Biomarine, Venice, California) as described [18], except that with some preparations, either 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.1 mg Trasylol per ml or 1 mM $CaCl_2$ were added during the initial homogenization. The middle band (37.5%) on the discontinuous sucrose gradient was used for all experiments. The membranes had an α-bungarotoxin binding activity of about 2 nmoles/mg protein.

The acetylcholine receptor was solubilized and purified essentially as described previously [10]. Membranes were diluted to 2.5 mg protein per ml in buffer I (60 mM KCl, 100 mM NaCl, 10 mM NaP_i, pH 8.0) and the pH adjusted to 10.6 with 1 N NaOH [12]. After 20 min at room temperature, they were centrifuged 20 min at 130,000g and the pellet was resuspended with buffer I to the original volume. K^+-cholate (20%) was then added to 1% final concentration. After 20 min at 4°C, insoluble material was removed by centrifugation at 130,000g for 20 min. The cholate extract was then added to 1/5 volume of packed choline carboxylmethyl affinity gel [7] and stirred gently for 2 h at 4°C. The gel was centrifuged at 750g for 2 min and washed five times with 20 volumes of buffer I containing 1% K-cholate and 1 mg crude soybean phospholipids per ml. The gel was then placed into a Pasteur pipette and the receptor was eluted with three column volumes of 10 mM carbamylcholine. The purified receptor was reconstituted into liposomes by the cholate dialysis procedure [10].

Exogenous Protease Treatments

Solubilized receptor was bound to the affinity gel and washed as described above. Various proteases were then added directly to the gel (100 μg protease per 1 mg acetylcholine receptor) in wash buffer and incubated 40 min on ice. The gels were then placed into Pasteur pipettes, washed with 50 volumes of the same buffer, and eluted with carbamylcholine as described above. This was sufficient to stop all proteolytic activity since 0 time points showed no degradation.

Ion Flux Measurements

Ion flux measurements were performed by adding 115 μl of reconstituted vesicles to 5 μl of 400 μCi ^{22}Na or ^{86}Rb per ml (New England Nuclear, Boston) plus 2.5 μl of H_2O or of 10 mM carbamylcholine. At 10 sec, 100 μl of the mixture was sampled onto a 3.8 ml Dowex column and then washed with 2 ml of 0.175 M sucrose [10]. The sample was collected in a scintillation vial with 10 ml of ACS scintillant and counted. For

measurement of desensitization, the carbamylcholine was added 20 sec before addition of ^{22}Na or ^{86}Rb.

α-Bungarotoxin Binding

N-[propionyl-^{3}H] α-bungarotoxin (Amersham, Arlington Heights, Illinois) binding measurements were assayed on DEAE paper according to Schmidt and Raftery [13].

SDS Gel Electrophoresis

Samples were prepared by dialyzing about 15 μg of protein against 0.2% SDS and 6.25 mM Tris-HCl (pH 6.8) for 12 h to remove cholate. They were then lyophilozed and resuspended in 100 μl of 5% SDS, 62.5 mM Tris-Cl (pH 6.8) 5% β-mercaptoethanol, 0.1 M dithiothreitol, and 10% glycerol, and incubated 2 h at 37°C. SDS gel electrophoresis was done essentially as performed by Laemmli [14]. The concentration of acrylamide in the separation gel was 13%.

Sucrose Gradients

Samples were incubated with a slight excess of [^{3}H] α-bungarotoxin in the presence of 10 mM β-mercaptoethanol and then layered on a 4.4 ml linear 5–20% sucrose gradient containing 1.0% K^{+}-cholate, 100 mM NaCl, 60 mM KCl, and 10 mM NaP_i (pH 8.0) in cellulose nitrate tubes. They were then centrifuged for 5 h in a Beckman SW 60 rotor at 58,000 rpm. Fractions (0.15 ml) were collected and aliquots of 0.1 were counted with 10 ml of ACS scintillant.

RESULTS AND DISCUSSION

Effects of Endogenous Proteases

Acetylcholine receptor purified either in the presence of protease inhibitors or in the presence of 1 mM Ca^{2+}, which activates endogenous proteases,* exhibit very different patterns in SDS electrophoresis gels (Fig. 1). The receptor prepared in the presence of Ca^{2+} reveals a pattern similar to that of the preparation of Changeux et al [9] with α as the major polypeptide. The receptor prepared in the absence of protease inhibitors has a diminished γ band. Yet all these preparations have similar α-bungarotoxin binding activities (8–9 nmoles/mg protein) and similar ion flux specific activities (1,600–1,800 nmoles/mg protein) which show the phenomenon of desensitization after reconstitution. Comparison of the carbamylcholine-dependent ion flux after reconstitution is a quantitative measure of the amount of functional receptor since it is directly proportional to the concentration of receptor.

Effect of Exogenous Proteases

In order to see how much degradation the receptor can tolerate we have exposed the acetylcholine receptor bound to an affinity column [7] to trypsin, chymotrypsin, pronase, and alkaline proteinase b. After reconstitution, the treated receptors still catalyzed carbamylcholine-dependent ion flux and showed the phenomenon of desensitization (Table I). The binding of α-bungarotoxin, although not measured in this experiment, always paralleled the ion flux activity. When analyzed on SDS gel electro-

*Hamilton S, Karlin A (personal communication).

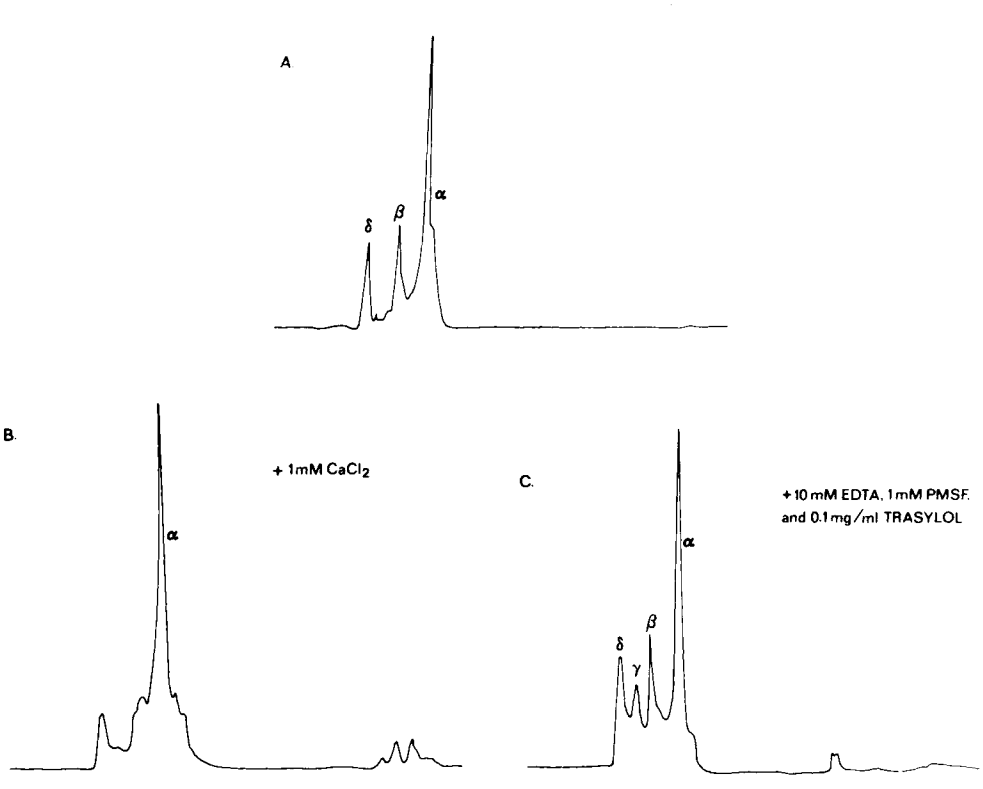

Fig. 1. Densitometer scan of SDS polyacrylamide gels of purified acetylcholine receptor purified from membranes prepared in the presence of A) no addition; B) 1 mM $CaCl_2$, and C) 10 mM EDTA, 1 mM PMSF, and 0.1 mg/ml Trasylol. SDS polyacrylamide gels were done as described in Materials and Methods.

TABLE I. ^{86}Rb Uptake Catalyzed by Protease-Treated Acetylcholine Receptors

	Ion uptake			
Sample	−CARB	+CARB	Δ	Desensitized[a] Δ
	nmoles/ ^{86}Rb × 10_s^{-1} × mg protein^{-1}			
Control	817	2,435	1,618 (100%)	164
Trypsin-treated	860	2,634	1,774 (110%)	382
Pronase-treated	770	1,854	1,084 (67%)	280
Chymotrypsin-treated	787	2,380	1,593 (98%)	195
Proteinase b-treated	865	2,461	1,596 (99%)	252

All protease treatments were done as described in Materials and Methods. Percent recovery is indicated in parentheses.

[a]Carbamylcholine was added 20 sec before ^{86}Rb was added. The data were calculated by subtracting the values for ion uptake in the absence of carbamylcholine.

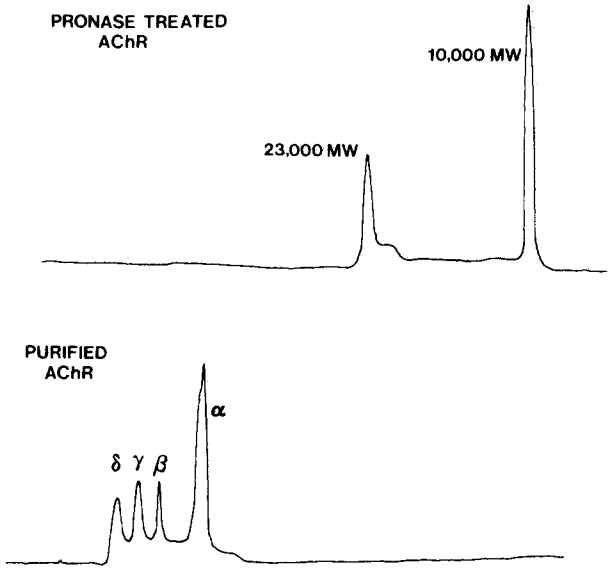

Fig. 2. Densitometer scans of SDS polyacrylamide gel of purified receptor and pronase-treated receptor. Receptor was purified from membranes prepared in the presence of 10 mM EDTA, 1 mM PMSF and 0.1 mg/ml Trasylol. SDS polyacrylamide gels were done as described in Materials and Methods.

TABLE II. Properties of ^{22}Na Uptake of the Pronase-Treated Acetylcholine Receptor

	Ion uptake				
Sample	−CARB	+CARB	Δ	Δ+CARB +2.5 μM α-bungarotoxin	Δ+CARB[a] +5 mM procaine
	nmoles $^{22}Na \times 10_s^{-1}$ mg protein^{-1}				
Control	569	2,215	1,646	173	132
Pronase-treated	313	1,389	1,076 (65%)	74	52

[a]Inhibitors were incubated 20 min at room temperature with reconstituted vesicles before assay. The data were calculated by subtracting the values for ion uptake in the absence of carbamylcholine.
Percent recovery is shown in parentheses.
α-Bungarotoxin binding specific activity of the control and pronase-treated receptor were 8.2 nmoles/mg protein and 4.6 nmoles/mg protein, respectively.

phoresis, the preparations appeared to be highly degraded. As shown in Figure 2, after treatment with pronase, only two bands of 23,000- and 10,000-dalton polypeptides were seen. This preparation still showed over 60% carbamylcholine-sensitive ion flux and sensitivity to α-bungarotoxin and procaine (Table II). In sucrose gradients (Fig. 3), the monomer complex appeared to be intact and sedimented similar to the untreated receptor with a sedimentation coefficient of 9S [7, 15].

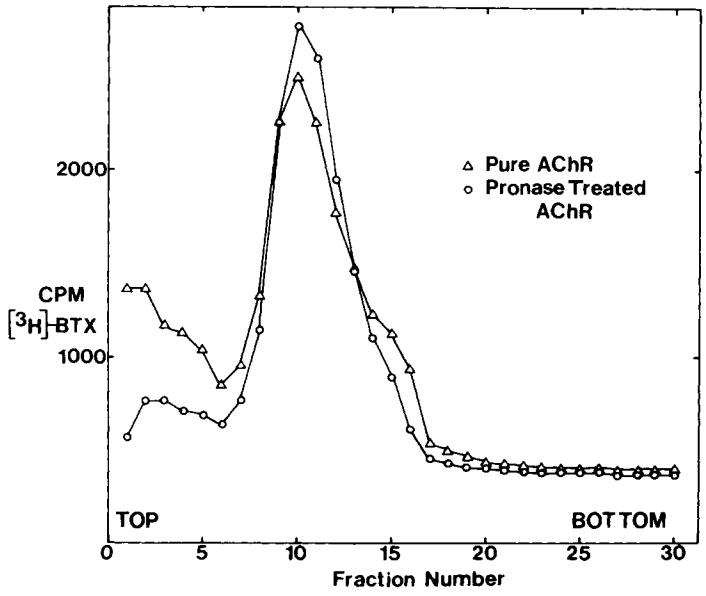

Fig. 3. Sucrose gradient centrifugation of reduced purified acetylcholine receptor and pronase-treated receptor ([^{3}H]-BTX = N-[propionyl-^{3}H] α-bungarotoxin). Sucrose gradients were performed as described in Materials and Methods.

These studies point to difficulties arising in the analysis of SDS acrylamide gels of proteins that have been nicked by endogenous proteases. Thus, variations in the subunit composition of the acetylcholine receptor reported in the literature [5, 8 –10, 16] may not reflect its true composition. When care was taken to purify the receptor in the presence of a mixture of protease inhibitors, acetylcholine receptors from such diverse species as rat, Torpedo, and Electrophorus electricus exhibit similar subunit composition [2, 17, 18].

Even greater caution is required in the interpretation of gels of proteins that have been exposed to protease. These observations need to be taken into consideration in immunological studies of protease-treated receptors [19] since treatment with papain yields preparations of the receptor that appear to have no apparent subunits on gels, yet retain antigenic sites for all four subunits and sediments at 9S [20].

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