CHARACTERIZATION OF A NICOTINIC ACETYLCHOLINE RECEPTOR FROM RABBIT SKELETAL MUSCLE AND RECONSTITUTION IN PLANAR PHOSPHOLIPID BILAYERS

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SUMMARY. The nicotinic acetylcholine receptor from rabbit skeletal muscle was isolated by affinity chromatography and characterized by ^{125}I- α -Bungarotoxin binding and gel filtration chromatography. Quantal conductance events were observed when this material was added to planar phospholipid bilayers. These changes were stimulated by carbamylcholine and antagonized by curare, Butx, dithiothreitol and concanavalin A. The receptor preparation was found to bind 0.2 nMoles ^{125}I- α -Bungarotoxin per mg protein and the molecular weight was estimated to be 390,000.

INTRODUCTION. Studies have been underway in several laboratories to find out if the isolated nicotinic acetylcholine receptor (AChR) contains a functional ion channel. By measuring electronic noise at the frog muscle endplate Katz and Miledi (1) have predicted that the conductance of the open AChR channel is about 10^{-10} mho. Fourier analysis of membrane current in voltage clamped endplates has yielded an estimate of 3.2×10^{-11} mho (2). We have measured discrete ion channel formation within this conductance range, in black lipid membranes doped with AChR isolated from mammalian brain by affinity chromatography (3, 4). This reconstituted membrane was sensitive to added cholinergic agents and exhibited multiple conductance events which suggested an orthogonal or tetrameric channel aggregation (5). Kemp et al. (6) reported the reconstitution of an AChR preparation obtained from both normal and denervated diaphragm muscle by 1.5% Triton X-100 extraction and gel filtration. This material induced conductance increases in egg phosphatidyl choline bilayers that were stimulated by acetylcholine but antagonized by α-Bungarotoxin (Butx) and curare. Changeux and his colleagues have shown that membrane fragments from Electrophorus (7) and Torpedo (8) containing the AChR, form sealed vesicles which transport Na^+ in response to added agonist. In fact, successful reconstitution in vesicles of native lipid has also been reported, after the dissolution of Torpedo membranes in sodium cholate (8) and after the purification of Torpedo AChR by affinity chromatography (9).

We now report the purification of AChR from rabbit skeletal muscle and its reconstitution in black lipid membranes.

MATERIALS AND METHODS.

Receptor Extraction. Naja naja siamensis α-cobrotoxin and α-bungarotoxin were obtained from the Miami Serpentarium. The preparation of the affinity gel using Affi-Gel (Bio-Rad Laboratories) has been described previously (5). Alternatively, pure α-cobrotoxin (0.25 mg/g gel) was bound to cyanogen bromide activated Sepharose 4B obtained from Pharmacia. Rabbit hindlimb muscle was placed in 0.5 M KCl, 50 mM sodium phosphate buffer, pH 7.4. The tissue was chopped in a Waring blender and homogenized in a motor driven glass homogenizer. The homogenate was centrifuged at 10,000 x g for 60 minutes and the pellet obtained was resuspended in 20 mM sodium phosphate, pH 7.4 containing 0.4% Triton X-100 and rehomogenized. This was then centrifuged at 13,000 x g for 60 minutes and the supernatant was applied to a neurotoxin affinity column as previously described (5). Some extractions utilized 0.4 mM phenylmethanesulfonyl fluoride as a protease inhibitor. The column was washed with 1 M NaCl, 50 mM sodium phosphate, pH 7.4, 0.1% Triton X-100 and AChR was eluted with 50 mM sodium phosphate, pH 7.4, 0.1% Triton X-100 and 1 M carbamylcholine chloride. The receptor was then dialyzed exhaustively against 0.1% Triton X-100, 50 mM sodium phosphate, pH 7.4. All procedures were carried out at 4°C.

Toxin Binding and Molecular Weight Determination. Butx was radioiodinated with Iodine-125, by the solid-state lactoperoxidase method of David and Reisfeld (10), using 1 mg Butx (0.13 μM), 0.13 μM KI, 0.3 mC Na ^{125}I, 0.2 μM H_2O_2 and 50 μg of Sepharose 4B-coupled lactoperoxidase. The reaction was stopped with sodium azide and the iodinated toxin was immediately loaded on a Sephadex CM-50 column (35 x 1.0 cm) equilibrated with 50 mM ammonium acetate, pH 5.8. The labeled toxin was eluted in a linear gradient to a final buffer concentration of 300 mM ammonium acetate, pH 7.0. The toxin eluted as a single peak and was pooled, lyophilized and stored in aliquots as an approximately 5 μM solution in 5 mM sodium phosphate, pH 7. The toxin had a specific activity of 1400 C/mole.

Routine assay for receptor was based on incubation with 13 nM ^{125}I-Butx for 60 minutes at room temperature and elution of the receptor-toxin complex from 2 cm x 0.5 cm CM-50 Sephadex columns in 0.1% deoxycholate-10 mM $NaPO_4$, pH 7.4.

Extensively dialyzed affinity column effluent was incubated with 0.2 nMoles of ^{125}I-Butx for 60 minutes at room temperature, the incubate was applied to a Sepharose 6B column (1.7 x 86 cm) and the sample was eluted in buffer containing 0.10% sodium deoxycholate. Five marker proteins of known molecular weight were chromatographed on the same column under identical conditions and a molecular weight estimate for the AChR: ^{125}I-Butx complex was determined by interpolation on a plot of log molecular weight vs. V_e/V_o. Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as a standard.

Bilayer Analysis. Lipid bilayers were formed using the methods first reported by Mueller et al. (12) Soybean lipid Type 11-S from the Sigma Chemical Company was dissolved in n-decane 30 mg/ml and then passed through columns of basic and acidic alumina prior to use. The bilayer was spread across a 1 mm aperature in a polyethylene chamber using a hollow polyethylene spreader filled with lipid as previously described (5). The aqueous phase consisted of 0.1 M NaCl. Calomel electrodes were used to apply a potential across the membrane and to measure the resulting current. A battery delivered 80 mV to the bilayer and the current generated were fed into a Keithley 427 amplifier. After the optically black configuration was achieved membranes were monitored for at least 60 minutes to insure baseline high resistance (10^{-9} mho cm^2). Samples of AChR up to 30 μl in buffer and 0.1% Triton X-100 were injected into the cis bilayer bath containing 5 ml of 0.1 M NaCl. For the investigation of pharmaco-

logical activity in the bilayer, all drugs used were injected into the bathing media on both sides of the membrane to produce the final concentration desired. In the case of dithiothreitol (DTT) receptor samples were first incubated for 6 hours in the presence of the stated concentration.

RESULTS. The use of cobrotoxin as an affinity ligand has been successfully applied to the purification of AChR electric fish (13) and mammalian diaphragm (14). Dialyzed affinity column effluent was labeled with ^{125}I-Butx and gel filtered on a Sepharose 6B column that had been calibrated with marker proteins. Interpolation in the plot in Figure 1 results in an estimated molecular weight of 390,000 for the AChR: Butx

Figure 1. A standard curve for estimation of molecular weight by gel chromatography on a Sepharose 6B column (86 x 1.7 cm). The samples were eluted at 4°C in a buffer compound of 100 mM NaCl; 2 mM histidine; 2 mM TES; 0.10% sodium deoxycholate, pH 7.4 at a flow rate of 5 ml/hr and 2 ml fractions were collected.

complex, in excellent agreement with values reported elsewhere (15). An analysis of the fractions eluted from the affinity column showed that both bilayer activity and toxin binding was found only in the early fractions eluted with carbamylcholine. The later fractions of this elution were completely inactive and did not demonstrate toxin binding. AChR purified by gel filtration retained full bilayer activity and toxin binding. Shortly after the dialyzed eluate was added to the inner bilayer chamber the steady-state conductance began to increase. Activity was only observed in thinned bilayers. There was some quantitative variability between preparations in both the amount of conductance induced and the retention of activity upon storage. The best preparations lost less than 10% activity in one month at 4°C. These conductance changes were composed of quantal events of several sizes and continuous variation was totally absent (Figure 2). The rise in conductance was a linear

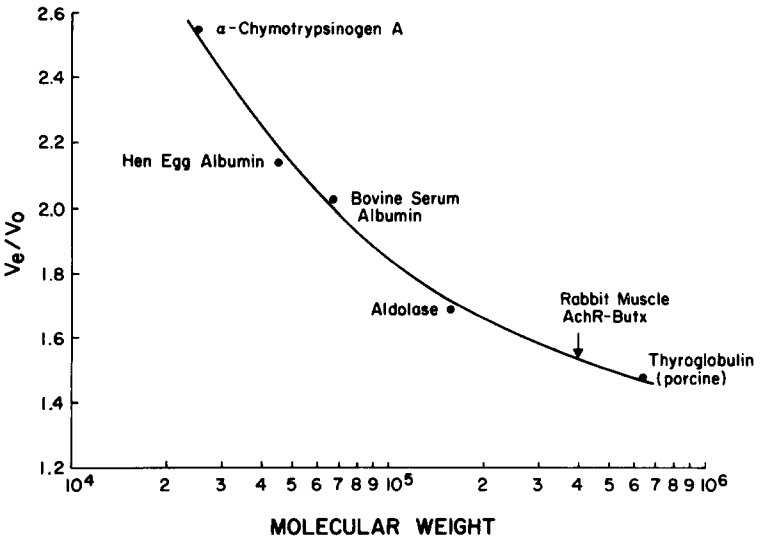

Figure 2. Continuous recording of a bilayer doped with muscle AChR.
Ordinate 10^{-9} mho, abscissa 30 sec
Rise time 100 msec - Keithley 427 current amplifier
Applied voltage 80 mV
The quantal events recorded here vary in size from 0.35×10^{-10} mho up to 1.3×10^{-9} mho.
Bathing solution 1M NaCl.

function of the amount of AChR added, in agreement with the findings of Kemp et al. (6). When carbachol was added to the bath, the conductance fluctuations were significantly increased as measured by rate of conductance development.

Table 1 describes the effects of several pharmacological agents on the rate of conductance development in the bilayer. Curare and Butx partially blocked the conductance increase after the addition of 30 μl AChR material in the presence of carbachol. Carbachol markedly enhanced the activity of small amounts (5 μl) of material which were otherwise only slightly active. Atropine had little effect on this preparation in contrast to the intact endplate where it is a moderate antagonist. Low concentrations of Con A had an antagonistic effect on conductance changes measured after addition of the AChR. Meunier et al. (16) have shown that Con A precipitates the AChR extracted from Electrophorus and inhibits the binding of radio active toxin to the receptor. Therefore the AChR may contain a glycoprotein which probably has terminal mannose or N-acetylglucosamine residues. However, as in the

Table 1. The effects of carbamylcholine and various ligands on the induced conductance. Rate is measured over the first ten minutes after addition of receptor preparation - expressed as mean ±S.E.M. C is 30 µl of a fraction later in the elution profile where no AChR is present.

µl of muscle AChR added to bilayer bath in the presence of 5 x 10^{-5} M carbamylcholine	Drug Added	Rate of Conductance Increase 10^{-10}mho min^{-1} (no. of replications)		
30	None	102.2	±8.4	(7)
30	1 x 10^{-4}M d-tubocurarine	33.0	±7.4	(4)
30	8 x 10^{-6}M α-bungarotoxin (Butx)	45.8	±7.3	(5)
30	6 x 10^{-4}M dithiothreitol (DTT)	4.0	±1.1	(4)
30	2 µg/ml concanavalin A (Con A)	9.1	±4.5	(3)
20	None	61.7	±3.8	(5)
20	1 x 10^{-3}M atropine	52.3	±9.4	(3)
5	None	8.7	±3.3	(3)
5	2.5 x 10^{-4}M carbamylcholine	81.8	±10.8	(4)
C	None	1.2	±0.4	(9)

case of the other drugs which we have tested, it is not yet possible to say whether Con A blocks the incorporation of channels into the bilayer or alters receptor function after incorporation. Incubation of the material with DTT also reduced activity significantly. There is biochemical evidence that the AChR from electroplax polymerizes by the oxidation of disulphide bonds (17). Our data may reflect the existence of receptor aggregates or polymers which are manifested by multiples of a basic conductance value or values. One of the actions of DTT might be to break down these polymers and thus reduce the probability of multiple events as we have, in fact, observed in the bilayer. In the electroplax AChR it has been shown that DTT decreases sensitivity to ACh by reducing a disulphide bond situated near the anionic site of binding (18). The noise recorded at the frog endplate after ACh application is also decreased after DTT treatment indicating that the active channel conformation may depend on a disulphide linkage (19). The further investigation of this phenomenon in the bilayer and its possible reversal by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) may be of some interest.

Figure 2 is a representative recording obtained after the addition of receptor material to the bilayer. Many different sizes of events are present and some of these, especially the smaller events, rapidly "turn on" and "turn off". The most frequent channel size is approximately 3.5×10^{-11} mho in 1 M NaCl, with other quantal events that appear to be multiples of this basic event. A similar fundamental event was found in neurotoxin binding fractions obtained from mammalian brain (5). Studies measuring bi-ionic potentials showed that this bilayer activity showed an Na^+/Cl^- selectivity of approximately 3:1 at pH 7.4.

It is possible that there is some relationship between the progression of conductance states observed here and the known polymerization or aggregation phenomenon reported for the isolated receptor (20). In support of this is the observation that freeze-fractured membranes from the neuromuscular junction (21) show orthogonal or rectangular arrays of particles. The role played by AChR aggregation in modulating the conductance, densitization, and cooperativity properties of the receptor may be of considerable importance.

Although it has recently been possible to purify the AChR in milligram quantities from electric fish, its functional reconstitution has proved to be a difficult problem. Reconstitution of the fish receptor by the authors has not been successful, and reports of success from other laboratories have been conspicuous in their absence. The only report of functional reconstitution, work which involved efflux of iostope from phospholipid vesicles (9), is by the authors' own admission only successful in some preparations. A more serious criticism is that the vesicle technique provides such limited information regarding the nature of the conductance change that it is not possible to judge whether the receptor is responding as it does in vivo. If the skeletal muscle receptor material were incorporated into vesicles (which is unfortunately not presently possible because of the large amounts of material required) and had an action on vesicles analogous to that reported here, the reconstitution would appear to be as successful as that reported for the fish receptor (9). It is only because of the greater flexibility of the planar bilayer technique that it is possible to determine whether such properties as agonist independent conductance,

channel magnitude and lifetime, etc. are inappropriate for the intact receptor.

Although the AChR used in this work is less pure than that reported for electric fish (9), the retention of the bilayer conductance behavior by such different techniques as gel filtration and affinity chromatography, in addition to its demonstrated drug specificity, strongly argues that the conductance changes are indeed a consequence of the AChR and are not some experimental artifact. Preliminary evidence suggests that modification of the conditions of receptor isolation and reconstitution can result in alterations in the nature as well as the magnitude of the conductance behavior. It is ultimately hoped that such investigations will result in the restoration of conductance properties consistent with those found in the post-synaptic neuromuscular junction.

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