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Comparison of the Subunits of *Torpedo californica* Acetylcholine Receptor by Peptide Mapping†

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ABSTRACT: The acetylcholine receptor from *Torpedo californica* electroplax was purified approximately 100-fold by affinity chromatograpy on α-neurotoxin–Sepharose 6B. Four putative subunits (α, β, γ, δ) of apparent molecular weights of 43 000, 52 000, 58 000, and 63,000 were found when the purified material was analyzed by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis. In some preparations, however, the amount of the γ polypeptide was small. The presence of *N*-ethylmaleimide throughout the purification procedure greatly enhanced the amount of the γ chain. To investigate the possibility that the putative subunits may be structurally related, they were isolated by preparative $NaDodSO_4$ gel electrophoresis and subjected to peptide mapping analyses. The patterns of fragments generated by *Staphylococcus aureus* V8 protease, papain, or chymotrypsin were different for each of the polypeptides. Thus, it is unlikely that they are derivatives of each other.

The acetylcholine (ACh)[1] receptor, the synaptic protein which binds ACh and mediates a membrane permeability change, has been the subject of intensive biochemical investigations in recent years. Neurotoxins, such as α-bungarotoxin, which bind the receptor with high affinity and specificity (Chang & Lee, 1973) and other affinity reagents have provided methods by which the receptor can be assayed and purified. A number of different laboratories have purified and characterized the receptor from the electric organs of the

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[1] Abbreviations used: ACh, acetylcholine; α-BuTx, α-bungarotoxin; NEM, *N*-ethylmaleimide; $NaDodSO_4$, sodium dodecyl sulfate; MBTA, 4-(*N*-maleimido)benzyltrimethylammonium iodide; BSA, bovine serum albumin; DDT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

0006-2960/79/0418-0301$01.00/0

marine ray, *Torpedo*, and the fresh water eel, *Electrophorus*, as well as from mammalian muscle. (See Heidmann & Changeux, 1978, for a recent review.)

There seems to be general agreement that the ACh receptor when solubilized with nondenaturing detergents is a large complex. When subjected to sucrose density gradient centrifugation, the *Torpedo* receptor behaves as a mixture of 9S monomer and 13S dimer (Potter, 1973; Raftery et al., 1975; Karlin et al., 1975; Gibson et al., 1976; Chang & Bock, 1977). The molecular weights have been determined to be 250 000 and 500 000 for the monomer and dimer, respectively, by sedimentation equilibrium analysis in which the contribution of bound detergent was blanked out by adjustment of the solvent density with D_2O (Reynolds & Karlin, 1978). This is in reasonable agreement with estimates made by gel electrophoresis after cross-linking (Biesecker, 1973; Hucho & Changeux, 1973) and by osmometry (Martinez-Carrion et al., 1975). Receptors from *Electrophorus* (Lindstrom & Patrick, 1974; Meunier et al., 1972; Karlin et al, 1975) and from a smooth muscle cell line consist entirely of 9S monomer, while purified receptor from skeletal muscle is almost all monomer (Froehner et al., 1977a). The function of the monomer–dimer relationship, which seems to be mediated by an intermolecular disulfide bond (Chang & Bock, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978), is unknown. Recent results suggest that *Torpedo* receptor exists in the membrane almost entirely as a dimer. Maintenance of the dimeric structure during solubilization and purification requires the presence of the sulfhydryl blocking reagent, *N*-ethylmaleimide (Chang & Bock, 1977).

The subunit structure of the ACh receptor is the subject of considerable disagreement. Receptors from various species of *Torpedo* purified by different protocols and analyzed by $NaDodSO_4$ gel electrophoresis have been reported to contain four polypeptides (α, β, γ, δ) of approximately 40 000, 50 000, 60 000, and 65 000 molecular weights (Weill et al., 1974; Raftery et al., 1975; Karlin et al., 1975; Hucho et al., 1976; Froehner et al., 1977a; Chang & Bock, 1977; Lindstrom et al., 1978). In some of the preparations, the amount of the γ chain was small relative to the other (Hucho et al., 1976; Froehner et al., 1977a; Lindstrom et al., 1978) or it was absent entirely (Penn et al., 1976). Other laboratories find two components (Heilbronn & Mattson, 1974) or only a single polypeptide of approximately 40 000 mol wt, presumably the α chain (Changeux et al., 1973; Potter, 1973; Carroll et al., 1973; Sobel et al., 1977). It has been suggested that the α chain interacts with the "ACh ionophore" (possibly a 43 000 mol wt polypeptide) in the membrane to form the functional unit (Sobel et al., 1978; Heidmann & Changeux, 1978).

The status of the subunit structures of the eel and the muscle ACh receptors is equally controversial. *Electrophorus* receptor has been reported to be composed of two subunits (43 000 and 48 000 mol wt) (Meunier et al., 1972), three subunits (40 000, 47 000, and 53 000 mol wt) (Karlin et al., 1975), and four subunits (48 000, 54 000, 60 000, and 110 000 mol wt) (Patrick et al., 1975). The receptor purified from denervated rat skeletal muscle is comprised of two major polypeptides of 45 000 and 51 000 mol wt plus other components of 49 000, 56 000, 62 000, and 110 000 mol wt (Froehner et al., 1977a). Boulter and Patrick purified the nicotinic ACh receptor from a nonfusing muscle cell line, BC_3H-1, and found four putative subunits of 44 000, 53 000, 65 000, and 72 000 mol wt. In contrast to these results, Merlie et al. (1978) found that the purest preparations of ACh receptor from primary cultures of fetal calf muscle are composed of a single class of polypeptide of 41 000 mol wt which exhibits some charge heterogeneity. Results similar to this were found with ACh receptor from denervated cat muscle. Preparations of high specific activity were composed almost entirely of a polypeptide(s) of 41 000 mol wt (Shorr et al., 1978).

Only one polypeptide has thus far been assigned a function. The α chain of *Torpedo* receptor and the corresponding smallest subunit of the eel receptor probably contain the acetylcholine binding site. They are specifically labeled with an affinity alkylating agent, 4-(*N*-maleimido)benzyltrimethylammonium iodide (MBTA), after reduction of the receptor with dithiothreitol (Karlin et al., 1975; Weill et al., 1974). Similar experiments with purified muscle receptor indicate that the smallest subunit of 45 000 as well as a polypeptide of 49 000 mol wt are both specifically labeled with [^{3}H]MBTA (Froehner et al., 1977b).

Since the functions of the other polypeptides are unknown, their authenticity as subunits of the ACh receptor remains undetermined. Even though the higher molecular weight chains are present in many preparations, the variability in the amounts of them suggests that their presence in a complex with the α subunit may be due to nonspecific interactions, possibly induced by solubilization with detergents (Sobel et al., 1977). Alternatively, the variability and even their absence from some preparations could result from proteolytic degradation or from weak association with the α subunit.

We report here a purification scheme for *Torpedo californica* ACh receptor that reproducibly yields a preparation composed of four polypeptides. The presence of NEM along with other protease inhibitors during the isolation procedure appears to be an important factor in achieving this result. Also we have isolated the individual putative subunits and subjected them to peptide mapping analysis. Since they appear to be unrelated polypeptides, it seems unlikely that they represent degradation products of each other.

Experimental Section

Materials

Torpedo californica electroplax was dissected out and frozen in liquid nitrogen by the supplier (Pacific Biomarine), shipped in dry ice, and stored at −80 °C. No detectable change in the receptor preparations was noted after storage of the tissue for up to 18 months. α-Bungarotoxin was prepared as previously described (Berg et al., 1972). [^{125}I]αBuTx was prepared according to the method of Vogel et al. (1972) and its specific radioactivity was determined by competition with αBuTx. Acrylamide and bisacrylamide were obtained from Bio-Rad Laboratories and $NaDodSO_4$ "Sequanal Grade" was purchased from Pierce. *Staphylococcus aureus* V8 protease was obtained from Sigma Chemical Co., and papain and chymotrypsin were from Worthington Biochemical Corp. Trasylol was purchased from Calbiochem.

Methods

ACh Receptor Assay. [^{125}I]αBuTx binding to solubilized receptor was determined with a DE81 filter assay as previously described (Froehner et al., 1977a,b) except that BSA at a concentration of 1 mg/mL was added to the reaction mixture. Binding activity in the crude homogenate was determined as described by Vogel & Nirenberg (1976).

Preparation of Affinity Resin. Cobrotoxin affinity resin was prepared as previously described (Brockes & Hall, 1975), except that Sepharose 6B instead of Sepharose 4B was used. The amount of active α-neutrotoxin per mL of Sepharose was estimated to be approximately 0.1 mg or about 20% of the input.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis was performed on slab gels using a procedure modified from Laemmli (1970). The sample buffer contained 2.5% $NaDodSO_4$ and 5 mM DTT. Samples were boiled for 5 min before electrophoresis. Bovine serum albumin (68 000), catalase (60,000), fumarase (49 000), ovalbumin (45 000), aldolase (40 000), glyceraldehyde phosphate dehydrogenase (36 000), and myokinase (21 000) (kindly provided by Dr. Lafayette Noda) were used as standards for molecular weight determinations (Weber & Osborn, 1969). Receptor and standards were mixed and applied to the same well for molecular weight determinations at different acrylamide concentrations. Gels were stained with Coomassie Brilliant Blue and destained according to Fairbanks et al. (1971).

Purification of Torpedo ACh Receptor. All procedures were carried out at 4 °C. Approximately 75 g of frozen tissue was sliced into small pieces with a razor blade and homogenized in 250 mL of 10 mM Tris-Cl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1 mM $PhCH_2SO_2F$, and Trasylol (10 units/mL) (buffer A) in a Waring blender. The homogenate was passed through a double layer of cheesecloth and centrifuged for 45 min at 27 000g. The pellet was resuspended in 25 mL of buffer A with a Dounce homogenizer, and Triton X-100 was added to a final concentration of 1%. After 1 h, insoluble material was removed by centrifugation (30 min at 27 000*g*), and the supernatant (Triton extract) was retained for affinity purification.

The Triton extract was stirred with 2–3 mL of cobrotoxin–Sepharose 6B (previously washed with buffer A containing 1 M NaCl and then equilibrated with buffer A) for 1.5–2 h. The mixture was then poured into a 10-mL plastic syringe with a nylon net support to form a column. The column was washed sequentially with 5 mL of buffer A containing 1.0% Triton X-100 (buffer B), 10 mL of buffer B containing 1 M NaCl, and 5 mL of buffer A containing 0.1% Triton X-100. Approximately 0.8 column volume of 1 M carbamoylcholine chloride in buffer A containing 0.1% Triton X-100 was run into the column. After 0.5–2 h, the first elution was collected. A second elution was collected after a further incubation of 12–15 h or, alternatively, the column contents were poured into 20–25 mL of carbamoylcholine solution and stirred slowly for 12–15 h. Eluted receptor was then collected after repouring the column. Both procedures for the second elution give comparable results. The affinity-purified fractions were pooled and then dialyzed against four changes of 500 mL of buffer A containing 0.1% Triton X-100 for at least 2 h each. For some preparations, 10 mM NEM was added to all buffers except the dialysis buffer which contained 1 mM NEM. Purified receptor stored at 4 °C showed no loss of [^{125}I]αBuTx binding activity and, except for some aggregation, no substantial change in its subunit structure over a period of several months. Receptor stored at −70 °C lost some binding activity after several freezings and thawings but no change in subunit structure was noted.

Isolation of ACh Receptor Subunits. ACh receptor (5–7 mg) prepared in the presence of 10 mM NEM was concentrated in a dialysis bag against sucrose to 4–7 mL and dialyzed against 0.125 M Tris-Cl, pH 6.8, 1 mM EDTA, 1 mM EGTA, 0.1 mM $PhCH_2SO_2F$, 0.1% Triton X–100. $NaDodSO_4$, DTT, glycerol, and bromphenol blue were added to final concentrations of 2.5%, 10 mM, 10%, and 0.001%, respectively. The sample was incubated for 20 min at room temperature and then applied to a 0.3 cm × 10 cm × 15 cm $NaDodSO_4$ slab gel (9% acrylamide). After electrophoresis, the protein bands were visualized by staining for about 5 min with 0.25% Coomassie Brilliant Blue, 25% isopropyl alcohol, 10% acetic acid. The gel was then destained for about 10 min with 25% isopropyl alcohol and rinsed briefly with water, and the bands were cut out with a razor blade. Elution of the protein from the gel was performed by electrophoresis. Gel slices were placed in disposable 10-mL plastic pipets fitted with small dialysis bags and filled with $NaDodSO_4$ reservoir buffer (0.05 M Tris, 0.38 M glycine, 0.1% $NaDodSO_4$, pH 8.3). Elution was performed at 5 mA per tube for 12–15 h in a Hoefer cylindrical gel apparatus (Hoefer Scientific Instruments, San Francisco, CA). Purified subunits were then dialyzed against 10 mM Tris-Cl, pH 7.4, 0.1% $NaDodSO_4$, and the protein concentrations were determined by the method of Lowry et al. (1951) using BSA as the standard. Purity of the subunits was assessed by $NaDodSO_4$ gel electrophoresis and, if necessary, the subunits were purified as described above. Subunits were stored at −70 °C.

Peptide Maps of Receptor Subunits. Purified subunits were analyzed by partial proteolytic digestion according to the procedure of Cleveland et al. (1977). Subunit protein (8 μg) was boiled for 2 min in a total volume of 31 μL of 0.125 M Tris-Cl, pH 6.8, 0.5% $NaDodSO_4$, 10% glycerol, 0.0002% bromphenol blue. Protease (5 μL) was then added. The final concentrations of each protease and times of incubation at 37 °C are indicated in the figure legends. The reaction was stopped by boiling for 3 min after the addition of 4 μL of 0.125 M Tris-Cl, pH 6.8, 20% $NaDodSO_4$, 10% glycerol, 0.004% bromphenol blue, 100 mM DTT. The samples were then subjected to gel electrophoresis on a 12% acrylamide $NaDodSO_4$ gel as described above.

Results

Affinity chromatography on cobrotoxin–Sepharose accomplishes the purification of the toxin-binding subunit(s) as well as other polypeptides associated with it. These may copurify with the toxin-binding subunit (presumably the α-subunit) due to artifactual formation of disulfide bonds during isolation and purification of the ACh receptor. To investigate this possibility, an excess of NEM was added during solubilization and the subsequent purification steps. Since NEM was found to have no effect on the extent of [^{125}I]αBuTx binding to the receptor (data not shown), purification could still be carried out by cobrotoxin affinity chromatography. ACh receptor was therefore prepared as described in Methods in the absence (normal preparations) and in the presence of 10 mM NEM (NEM preparations). NEM affected neither the yield of receptor obtained nor its specific activity. In 12 different preparations of ACh receptor (5 in the presence of 10 mM NEM), the yield of receptor from the cobrotoxin column averaged 52% (range of 33–72%). The best yields were obtained when the amount of cobrotoxin–Sepharose used was only in slight excess over the amount needed to absorb all of the receptor in the Triton extract. Most of the receptor was recovered in the first elution, although a second elution for an extended period usually yielded about 50% more. The specific activity of these preparations ranged from 5.1 to 12.2 nmol of [^{125}I]αBuTx bound per mg of protein with an average of 7.6 for seven preparations. This variability probably reflects inaccuracies in the determination of the specific radioactivity of the [^{125}I]αBuTx.

To investigate the subunit structures of these preparations, the purified receptor was denatured in $NaDodSO_4$ and subjected to slab $NaDodSO_4$ gel electrophoresis. Normal receptor preparations contained four polypeptides of apparent molecular weights of 43 000 (α), 52 000 (β), 58 000 (γ), and 63 000 (δ) when denatured in the presence of DDT (Figure

Table I: Dependence of Apparent Molecular Weights of Subunits on Acrylamide Concentration

concn	app mol wt			
	α	β	γ	δ
normal preparation				
7%	42 300	51 100	58 600	65 100
8%	43 000	52 300	59 000	64 800
9%	43 100	52 200	57 900	63 500
10%	43 700	52 700	58 900	63 700
11%	44 500	53 500	58 900	63 600
mean (± SD)	43 320 (±825)	52 360 (±870)	58 660 (±450)	64 140 (±750)
K_R vs. M_R	48 200	59 000	61 500	63 900
NEM preparation				
7%	45 100	50 900	60 300	65 200
8%	46 000	52 800	60 800	65 100
9%	45 900	53 000	60 500	64 500
10%	46 600	53 300	60 300	64 100
11%	46 200	53 500	60 200	63 400
mean (± SD)	45 980 (±554)	52 700 (±1041)	60 400 (±200)	64 460 (±744)
K_R vs. M_R	46 800	62 400	64 600	67 100

1A). In six out of seven preparations, the γ polypeptide was the least predominant and appeared on the gel as a diffuse indistinct band. Its amount relative to other chains was extremely variable and in some cases it was comprised of two faint bands. If NEM was present during the preparation, a similar pattern of polypeptides was found with two major differences (Figure 1B). The apparent size of the α chain was about 46 000 and the γ chain migrated as a sharp, distinct, single band of approximately 60 000. The behavior of the β and δ chains was relatively unaffected. A minor component was also present between the α and β polypeptides. Antisera raised against a normal preparation of receptor reacts with all four polypeptides of both normal and NEM preparations (data not shown). Thus, the polypeptides present in NEM preparations are the same ones found in normal preparations.

The apparent increase in size of the α chain in NEM preparations is probably a direct effect of the alkylating agent. Conversion of the 43 000 mol wt α subunit of normal preparations to 46 000 could be accomplished by treatment with 10 mM NEM, either before or after reduction with DTT. Lower concentrations of NEM were less effective, usually yielding a mixture of 43 000 and 46 000 mol wt polypeptides. Since the α chain incorporates much more [^{3}H]NEM when reacted with 10 mM than when treated with 1 mM (data not shown), it seems likely that the decreased mobility of the α subunit in NEM preparations is a consequence of the greater degree of alkylation that occurs with 10 mM NEM. Similar effects on the mobility of other proteins have been noted (Lane, 1978).

In contrast, the changes in the behavior of the γ chain on $NaDodSO_4$ gels could not be accomplished by direct treatment with NEM. A sharp distinct polypeptide was present in all five NEM preparations but in only one of the seven normal preparations. In this one case, the amount of the γ chain appeared to be approximately one-third to one-half that found in NEM preparations. Thus, NEM is required during the isolation and purification procedure to obtain reproducible amounts of the γ chain. Its mechanism of action may be to prevent proteolysis, either by the inhibition of a thiol protease or by the stabilization of intramolecular disulfide bonds in a manner similar to that proposed for the preservation of the disulfide bond responsible for dimerization of the receptor (Chang & Bock, 1977). Reduction of intramolecular disulfides may cause a conformational change in the polypeptide, rendering it susceptible to proteolytic degradation.

If the amount of each of the polypeptides is estimated from densitometer tracings of Coomassie Blue stained gels, the mass

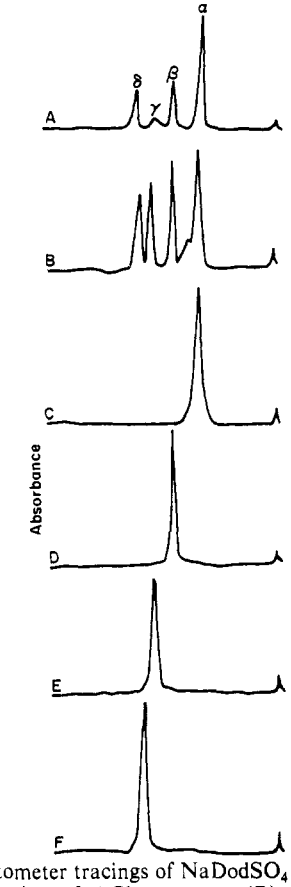

FIGURE 1: Densitometer tracings of $NaDodSO_4$ gels of (A) 5 μg of a normal preparation of ACh receptor, (B) 4 μg of an NEM preparation of ACh receptor, (C) 1 μg of subunit α isolated from a preparative $NaDodSO_4$ gel, (D) 1 μg of subunit β, (E) 0.7 μg of subunit γ, and (F) 1 μg of subunit δ.

ratios of α:β:γ:δ are approximately 1.0:0.45:0.2:0.35 for normal preparations and 1.0:0.5:0.4:0.5 for NEM preparations. The latter suggests that the 9S complex is composed of 2–3 mol of α and 1 mol each of β, γ, and δ. Reynolds & Karlin (1978) suggest that a molar stoichiometry of 2:1:1:1 is consistent with a molecular weight of 250 000 for the 9S monomer.

For both normal and NEM preparations, the apparent molecular weights of the subunits appear to be independent of the gel acrylamide concentration when derived by plotting relative mobility vs. log molecular weight (Table I). However, when molecular weights were extrapolated from plots of the retardation coefficient, K_R (derived from Ferguson plots), vs. the molecular weight (Frank & Rodbard, 1975), differences

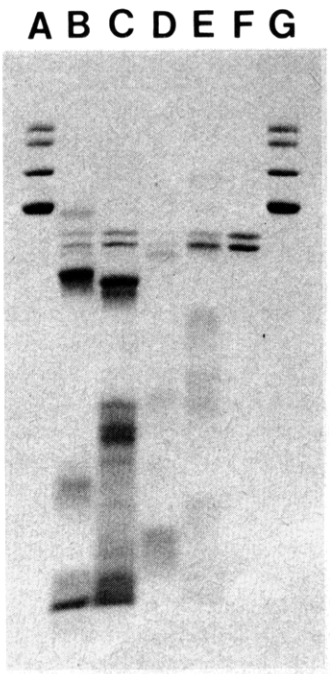

FIGURE 2: Peptide maps of purified subunits (8 μg) after digestion with *Staphylococcus aureus* V8 protease for 60 min at 37 °C. (A & G) contain 5 μg of an NEM preparation of ACh receptor to indicate the position of the undigested subunits, (B) subunit α digested with 2.2 μg of protease, (C) subunit β digested with 3.3 μg of protease, (D) subunit γ digested with 0.9 μg of protease, (E) subunit δ digested with 2.5 μg of protease, and (F) 3.3 μg of protease incubated alone.

were noted for α and β of normal preparations and for β of NEM preparations. These differences, though not very large, suggest that these polypeptides migrate anomalously on $NaDodSO_4$ gels.

When NEM is present in excess throughout the purification, including the initial homogenization of the tissue, any disulfide bonds present in the receptor should exist as such in the membrane. If the purified receptor was denatured in $NaDodSO_4$ (in the presence of 10 mM NEM to block any sulfhydryl groups exposed by denaturation) and subjected to $NaDodSO_4$ gel electrophoresis without prior reduction, the δ chain of NEM preparations is almost entirely replaced by a polypeptide of approximately 130000, presumably a dimer of the δ chain, while the α, β, and γ chains migrate as in the reduced sample. Results similar to this have been previously reported (Chang & Bock, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978).

The susceptibility of the ACh receptor to proteolytic degradation after solubilization with detergents (Patrick et al., 1975; Raftery et al., 1975) raised the possibility that the putative subunits may represent degradation products. To determine the relationships among the polypeptide chains, we have isolated them from $NaDodSO_4$ gels and subjected them to peptide mapping by limited proteolysis in $NaDodSO_4$ as described by Cleveland et al. (1977). Subunits from ACh receptor prepared in the presence of NEM were separated on a preparative $NaDodSO_4$ gel and recovered from the gel electrophoretically. Recovery of the subunits was estimated to be 30–50%. Subunits isolated by this procedure remain intact and are free of any contamination by other subunits (Figure 1). Studies of the minor component intermediate in mobility to α and β (Figure 1B) were precluded by the small quantities present.

Peptide maps of each of the subunits digested with *Staphylococcus aureas* V8 protease are shown in Figure 2. The concentrations of protease were chosen so as to leave only a small amount of each subunit intact, the position of which can be seen by comparison with undigested whole receptor (Figures 2A and 2G). Differing susceptibilities of the subunits to proteolysis in $NaDodSO_4$ necessitated the use of different concentrations of protease. To control for autodigestion, the protease was incubated alone at the highest concentration used for digestion of subunits and was subjected to electrophoresis. Only the intact protease, seen as a doublet slightly smaller than the α chain of the receptor, is present (Figure 2F). A number of fragments are generated by digestion of the subunits, ranging in size from slightly smaller than the intact subunit to about 8000. The pattern of the fragments is different for each subunit, suggesting that there are no major similarities in their primary structures.

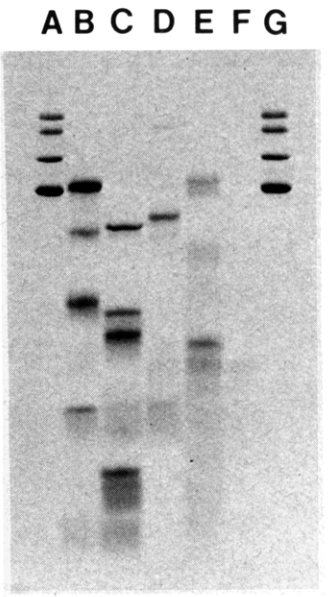

FIGURE 3: Peptide maps of purified subunits after digestion with papain for 30 min at 37 °C. (A & G) contain 5 μg of undigested ACh receptor, (B) subunit α digested with 0.20 μg of papain, (C) subunit β digested with 0.125 μg of papain, (D) subunit γ digested with 0.20 μg of papain, (E) subunit δ digested with 0.375 μg of papain, and (F) 0.375 μg of papain incubated alone.

A similar experiment in which the subunits were digested with papain is shown in Figure 3. Again, the patterns of fragments produced by partial proteolytic cleavage are different for each of the four subunits. Peptides of similar mobility are present in the digests of α (Figure 3B) and γ (Figure 3D) at positions just below that of papain (Figure 3F). Since the fragments are resolved only according to size, similar mobilities do not necessarily indicate sequence homologies. The absence of any other similarities between the digest of α and γ argues against any substantial homology.

Digestion of the subunits with chymotrypsin (Figure 4) is complicated by the large amount of protease required and by protease autodigestion. The lower 20% of the gel is obscured by the enzyme and its autodigestion fragments (Figure 4F). However, a large number of fragments are resolved above this region and again the patterns are different for each of the subunits.

Discussion

The procedure described here for the purification of ACh receptor is a simple one that can easily be performed in 2 days, is reproducible, and can be scaled up for the preparation of larger amounts of receptor. In contrast to results with receptor from muscle (Froehner et al., 1977a,b; Merlie et al., 1978), the yields of activity from the α-neurotoxin column are quite high and reproducible. The higher yields obtained may reflect

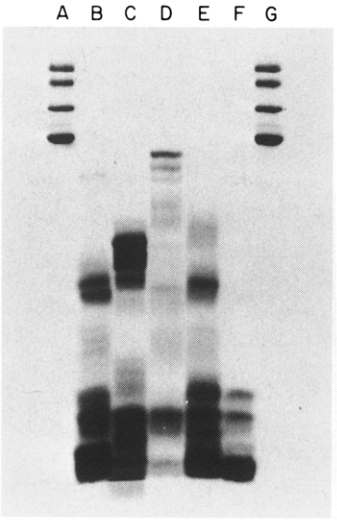

FIGURE 4: Peptide maps of purified subunits after digestion with chymotrypsin for 30 min at 37 °C. (A & G) contain 5 μg of undigested ACh receptor, (B) subunit α digested with 5 μg of chymotrypsin, (C) subunit β digested with 2.5 μg of chymotrypsin, (D) subunit γ digested with 1.5 μg of chymotrypsin, (E) subunit δ digested with 5 μg of chymotrypsin, and (F) 5 μg of chymotrypsin incubated alone.

the larger ratio of receptor to α-neurotoxin–Sepharose, a condition that seems to favor better recovery at this purification step (Merlie et al., 1978).

The subunit structure that we report here is similar to that described by several other laboratories (Weill et al., 1974; Raftery et al., 1975; Karlin et al., 1975; Hucho et al., 1975; Chang & Bock, 1977; Froehner et al., 1977a,b; Lindstrom et al., 1978). The inclusion of NEM clearly is an important factor in obtaining consistent subunit patterns with this procedure. If the effect of the alkylating agent is to inhibit proteolysis, then this may offer at least a partial explanation for the absence of the γ polypeptide from some preparations (Penn et al., 1976; Heilbronn & Mattson, 1974; Changeux et al., 1973). It should be noted that the other protease inhibitors used may not be sufficient to prevent degradation. In particular, the instability of $PhCH_2SO_2F$ in aqueous solutions limits its usefulness in this regard (James, 1978). NEM also prevents the artifactual formation of disulfide bonds during the purification of the receptor as indicated by the results of $NaDodSO_4$ gel electrophoresis without prior reduction. The only intermolecular disulfide bonds in the receptor complex are between two δ chains. As previously shown (Chang & Bock, 1977; Hamilton et al., 1977), these are most likely responsible for the dimerization of the receptor and are stabilized by the presence of NEM during the isolation. We have confirmed the result that NEM preparations are approximately 95% dimeric and find that all four polypeptides are present in the 13S form in approximately the same ratio as shown in Figure 1B. Normal preparations were composed of 50–95% monomer (J. Caughey & S. Froehner, unpublished results).

The technique of peptide mapping by limited proteolysis in $NaDodSO_4$ is particularly well-suited to analysis of the putative subunits of the ACh receptor. Only small amounts of each polypeptide are needed and problems of insolubility of membrane proteins are avoided by performing the digestions in $NaDodSO_4$. Digestion with three proteases of different specificities revealed no similarities in primary structure among the four polypeptides. The same conclusion has been reached independently by another laboratory using a procedure similar to the one described here (N. Nathanson & Z. Hall, personal communication). This is in agreement with immunological studies in which antisera to the individual polypeptides reacted only with the subunit used as the immunogen and not with any of the others, both when analyzed by double immunodiffusion (Claudio & Raftery, 1977) and by a competition radioimmune assay (Lindstrom et al., 1978).

Since the fragments were compared only on the basis of size, short regions of homology could exist which would not be detected by this technique. For instance, if binding sites for α-neurotoxin are present on two different subunits (Hamilton et al., 1978), then similar or possibly identical primary sequences could exist. Such similarities probably would not have been detected. Cleveland et al. (1977) demonstrated a large number of common peptides between protein P23*, the 45 000 mol wt major head protein of bacteriophage T4, and its 55 000 molecular weight precursor, P23, by this technique. Some bands unique to the precursor were also found. The possibility that one or more of the receptor polypeptides represent precursors or degradation products of others appears unlikely since no common peptides were found. However, the possibility that some or all are derived from still larger polypeptides is not eliminated. Furthermore, their authenticity as components of the ACh receptor awaits determination of their functions.

Acknowledgments

We wish to thank Dr. Melitta Schachner for her interest in and support of this project and Drs. Nathanson and Hall for communicating their results to us prior to publication. A portion of the results presented here comprised an undergraduate honors thesis for S.R. in the Department of Biochemistry and Molecular Biology, Harvard University.

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Selective Phosphorylation of a Nuclear Envelope Polypeptide by an Endogenous Protein Kinase†

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ABSTRACT: Incubation of highly purified nuclear envelope with [γ-^{32}P]ATP resulted in the selective phosphorylation of a major membrane polypeptide (M_r 68 000) by an endogenous protein kinase. Phosphorylation of this major polypeptide decreased drastically upon perturbation with low concentrations of Triton X-100 or sodium deoxycholate. Similar results were obtained when the nuclear envelope was sonicated vigorously prior to incubation with [γ-^{32}P]ATP. No stimulation of ^{32}P incorporation was noted in the presence of either cAMP or cGMP. Magnesium ion was required for maximal phosphorylation of the 68 000 molecular weight polypeptide; however, Mn^{2+} was approximately 40% as effective as Mg^{2+}. No phosphorylation occurred in the presence of Ca^{2+} or Cu^{2+}. Optimal phosphorylation was obtained between pH 6 and 9. Studies with the membrane matrix of the endoplasmic reticulum revealed the complete absence of the readily labeled 68 000 molecular weight polypeptide found in the nuclear envelope. Phosphorylation of the microsomal membrane was less specific as evidenced by the incorporation of ^{32}P into at least three proteins, the most prominent having molecular weights of 54 000 and 49 000. Both membranes yielded *O*-phosphoserine and *O*-phosphothreonine in a ratio of 3:1 after acid hydrolysis. Partially purified preparations of pore complexes derived from nuclear envelope showed an enrichment of the 68 000 molecular weight protein, suggesting the possible involvement of this highly selective kinase reaction in the exchange of solutes between the nucleus and cytoplasm.

The nuclear envelope, composed of an inner and outer leaflet, each of which possesses the morphological characteristics of a typical membrane, forms the physical barrier separating the nucleoplasm from the remainder of the cell. A unique structural feature of the envelope is the pore complex which, although it is an integral part of the envelope, does not appear to possess the basic phospholipid bilayer structure common to the remainder of the envelope. The physical and biochemical characteristics of the nuclear envelope have been recently reviewed (Kay & Johnston, 1973; Kasper, 1974a,b; Franke & Scheer, 1974; Wunderlich et al., 1976; Harris, 1978), and it is apparent that an integrated picture of structure and function is slowly beginning to emerge. For example, it is clear that the polypeptide composition of the nuclear envelope differs significantly from that of the microsomal membrane (Bornens & Kasper, 1973) in spite of the fact that the enzymology of these two closely associated membrane systems is qualitatively very similar. Also of functional importance is the fact that the nuclear envelope TPNH

† From the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706. *Received August 11, 1978.* Supported by Grants CA-17300 and CA-07175 from the National Cancer Institute.

0006-2960/79/0418-0307$01.00/0