Mammalian Muscle Acetylcholine Receptor Purification and Characterization†

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ABSTRACT: Nicotinic acetylcholine receptor (AcChR) was purified from fetal calf muscle by an affinity chromatographic method utilizing α-neurotoxin from *Naja naja siamensis* as an immobilized ligand. Preparations of AcChR with an average specific activity of 6 nmol of α-toxin bound/mg of protein were obtained, i.e., 75% of the theoretical specific activity assuming identity with *Torpedo* AcChR. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified AcChR consistently showed the presence of five polypeptides, having apparent M_r's of 42 000, 44 000, 49 000, 55 000, and 58 000, respectively. The peptide of M_r 44K was demonstrated to be actin. The amino acid composition of fetal calf AcChR was shown to be similar to that of *Torpedo* AcChR. In addition, calf AcChR contained large amounts of amino sugars. The sedimentation coefficient of the purified calf AcChR was found to be 9.25 ± 0.25, i.e., similar to the monomeric form of electric organ AcChR. Determination of the isoelectric point of α-bungarotoxin/calf AcChR complexes revealed the presence of two charged forms, having p*I* values of 5.16 ± 0.13 and 6.05 ± 0.18, respectively.

The nicotinic acetylcholine receptor (AcChR)[1] from *Torpedo* electric organ has been purified and extensively characterized [reviewed in Heidmann & Changeux (1978) and Conti-Tronconi & Raftery (1982)]. It is now agreed that it is a pentameric pseudosymmetric complex (Raftery et al., 1980) of four different subunits, one of which is present in two copies (Lindstrom et al., 1979a; Raftery et al., 1980; Strader et al., 1980).

Less agreement exists regarding the structure of the mammalian muscle nicotinic AcChR. This is mainly due to the fact that the isolation of muscle AcChR has been difficult compared with the relatively facile purification of solubilized preparations from electric ray electroplax tissue, due both to the lower AcChR content (Potter, 1973; Nathanson & Hall, 1980) and to the much higher protease activity of muscle than of electric organ (Einarson et al., 1982). Conflicting reports about the subunit composition of the mammalian AcChR have appeared, the preparations reported being composed of from one (Merlie et al., 1978; Shorr et al., 1978; Lyddiatt et al., 1979) to six (Froehner et al., 1977; Boulter & Patrick, 1977; Nathanson & Hall, 1979; Merlie & Sebbane, 1981) protein components. To date no method has been described for purification of mammalian AcChR in amounts sufficiently large to allow definition of its exact subunit complement and characterization of its subunits. This is critical for clarification of its structure since, by analogy with the *Torpedo* AcChR, chemical characterization of the receptor subunits to the level of their NH_2-terminal sequences has proven to be the only reliable method for the unequivocal identification of the peptides which are part of the AcChR complex (Raftery et al., 1980).

In this paper we describe a method for purifying large amounts (nanomoles) of mammalian AcChR which yields a complex subunit pattern in $NaDodSO_4$ gel electrophoresis, similar to *Torpedo* AcChR, and the elucidation of some of its chemical and physical properties.

Experimental Procedures

Materials

Fetal calves were obtained locally. Lyophilized *Naja naja siamensis* venom was obtained from Biotoxins Corp. ^{125}I-Labeled α-bungarotoxin was obtained from New England Nuclear. DE-81 disks were from Whatman Ltd. Sepharose 2B, carbamoylcholine, protein A, and concanavalin A were obtained from Sigma Chemical Co. Ampholines for isoelectric focusing were purchased from LKB. Anti-actin antiserum was the generous gift of Dr. Elias Lazarides. Anti-rabbit immunoglobulin antiserum was the generous gift of Dr. Jon Lindstrom. Reagents for gel electrophoresis were obtained from Bio-Rad Laboratories. All other chemicals were of the highest purity commercially available.

Methods

Neurotoxin Purification and Coupling to Agarose Beads. α-Bungarotoxin (α-BuTx) was purified according to Clark et al. (1972). *N. naja siamensis* α-neurotoxin was purified from lyophilized venom as described by Ong & Brady (1974). The peptide composition of the column fractions was assessed by $NaDodSO_4$–polyacrylamide gel electrophoresis according to Laemmli (1970) using slab gels (1 mm × 13 cm × 2 cm) containing an exponential gradient of acrylamide from 15% to 22.5% and 0.1% $NaDodSO_4$. The stacking gel contained 4% acrylamide. The purity of the isolated toxin was assessed by NH_2-terminal amino acid sequencing. After purification the toxin was coupled to Sepharose 2B as described by Porath et al. (1973). The capacity of the resin was determined by using an excess of *Torpedo* AcChR.

Purification of AcCh Receptor. All procedures were carried out at 4 °C. Muscles from fetal calves (10–15 in. long) were immediately dissected and homogenized in 1 volume of 50 mM

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[1] Abbreviations: AcChR, acetylcholine receptor; Carb, carbamoylcholine; α-BuTx, α-bungarotoxin; buffer I, 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, and 1.2 mM EGTA; buffer II, 10 mM sodium phosphate, pH 7.4, 50 mM NaCl, and 0.1% Triton X-100; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; IAA, iodoacetamide; $NaDodSO_4$, sodium dodecyl sulfate; Gal-NH_2, galactosamine; Glu-NH_2, glucosamine; PMSF, phenylmethanesulfonyl fluoride.

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Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, and 1.2 mM EDTA (buffer I) containing 40 mM PMSF. The homogenate was centrifuged at 24000*g* for 150 min. Pellets were resuspended in 1 volume of buffer I with 20 mM PMSF and 10 mM IAA and stored at −70 °C. The frozen pellets were homogenized in a Waring blender for 1 min at high speed with 1 volume of buffer I containing 2 mM PMSF, 5 mM IAA, and 2% Triton X-100 and extracted for 1 h at 4 °C with moderate stirring.

The extract was centrifuged at 24000*g* for 80 min. The pellets and the upper part of the supernatant containing lipids were discarded, and the clear supernatant was adsorbed on the affinity resin (50 mL for an amount of Triton extract equivalent to 1500 g of starting muscle; capacity 0.03–0.05 mg of AcChR/mL of settled resin) packed in a 150-mL glass Büchner. The flow rate was 400 mL/h, and the extract was passed twice through the resin. The resin was washed with 20 volumes of buffer I containing 1% Triton X-100, 40 volumes of buffer I containing 1% Triton and 1 M NaCl, and 20 volumes of buffer I containing 0.1% sodium cholate. After the column was washed, 1 volume of 1.5 M carbamoylcholine (Carb) in buffer I containing 0.1% sodium cholate was added to the column and left for 10 h. The Carb eluate was centrifuged at 250000*g* for 3.5 h in a Beckman Ti 75 rotor. The resulting pellet was resuspended in 10 mM sodium phosphate buffer, pH 7.4, 50 mM NaCl, and 0.1% Triton X-100 (buffer II), centrifuged for 5 min in a Beckman airfuge to eliminate any debris, and dialyzed vs. wash buffer (AcChR I).

The affinity resin was further incubated with 1 volume of 52.5 mM Tris-HCl, pH 6.8, 5% mercaptoethanol, and 3% $NaDodSO_4$ for 60 min at room temperature. This eluate was extracted with 2 volumes of chloroform and dialyzed against 32 mM Tris-HCl, pH 6.8, containing 0.1% $NaDodSO_4$ (AcChR II).

Torpedo AcChR was purified from electric organs by affinity chromatography.

Protein Determination. Protein concentration was determined according to Lowry et al. (1951) by using BSA as a standard. The standard contained Triton X-100 at the same concentration as the sample. The precipitate observed upon addition of the Folin reagent was removed by centrifugation at 5000 rpm for 5 min prior to reading the absorbance at 750 nm.

AcChR Assay. [^{125}I]-α-BuTx binding to crude homogenate and to solubilized receptor was determined with the DE-81 DEAE disk assay as previously described by Schmidt & Raftery (1973), using 10^{-8} M [^{125}I]-α-BuTx and 4–12-h incubation at room temperature.

Density Gradient Centrifugation of [^{125}I]-α-*BuTx–AcChR.* Linear gradients from 5% to 20% (w/v) sucrose in buffer II were prepared by using a Beckman gradient maker and were kept at 4 °C for 6 h. The volume of each gradient was 12 mL. AcChR I (500 μL) was incubated with [^{125}I]-α-BuTx for 12 h at 4 °C, loaded on the gradient, and centrifuged for 17 h at 40 000 rpm in a Beckman SW 40.1 rotor. The nonspecific binding was determined by preincubation of AcChR I with an excess of cold α-BuTx. Fractions of 0.2 mL were collected from the bottom of the tube and counted in a Beckman Biogamma II counter.

Amino Acid Analysis. Aliquots of AcChR I (7–12 μg) were hydrolyzed in vacuo with 6 N HCl at 110 °C for 24 h. Hydrolysates were vacuum dried and analyzed on a Beckman 120B amino acid analyzer.

Isoelectric Focusing. Isoelectric focusing of AcChR I–[^{125}I]-α-BuTx complex was performed as previously described (Brockes & Hall, 1975) with the following changes. AcChR–[^{125}I]-α-BuTx complexes were formed during 5-h incubation at 4 °C. Gels (5.5 mm × 11.5 cm) were prerun for 60 min at 1 mA/gel. Focusing was carried out for 12 h at 200 V plus 1 h at 400 V. The gel slices (4 mm) were counted in a Beckman Biogamma II counter.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970) by using slab gels containing 8.75% polyacrylamide. *Torpedo* AcChR subunits were used as standards.

Two types of slabs were used, both 1 mm thick, and with a running gel 14 cm long. The spacer gel in one type (used for AcChR I) was 2 cm long, while in the second type (used for AcChR II) it was 14 cm long. The volume left for the sample was 230 μL/cm of gel width in the first case, and 10 times as much in the second case. Sodium thioglycolate (1 mg/mL) was added to the samples, and the gels were electrophoresed at 20 mA. The gels were stained with Coomassie Brilliant Blue according to Fairbanks et al. (1971), and strips were scanned at 550 nm by using a Gilford Model 240 spectrophotometer equipped with a linear transport unit.

Precipitation of Purified AcChR by Anti-antigen Antibodies. A 200-μL sample of solubilized AcChR ($\sim 10^{-8}$ M) was incubated with 20-fold excess of [^{125}I]-α-BuTx for 6 h at room temperature. Twenty microliters of anti-actin antiserum was added (or preimmune serum), and the mixture was incubated overnight at 4 °C. An optimal amount of anti-rabbit antiserum containing 1% Triton X-100 was added. The mixture was incubated for 4 h at room temperature, spun down, washed twice with buffer I, and counted in a Beckman scintillation counter.

Results

Purification of α-Toxins and Their Coupling to Sepharose 2B. The peptide composition of α-BuTx, purified by two cation-exchange chromatography steps, was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and was found to be composed (to >95%) of a peptide of the expected molecular weight, i.e., M_r 8K. *Naja naja siamensis* α-neurotoxin was also purified by two cation-exchange chromatographic steps, and after each chromatography the peptide composition of each fraction was determined by $NaDodSO_4$–polyacrylamide gel electrophoresis. After the second column only those fractions that contained the α-neurotoxin free of contaminants were used. The purity of the pooled fractions was further assessed by NH_2-terminal amino acid sequence analysis, and the sequence found corresponded to that reported for *N. naja siamensis* α-neurotoxin (Karlsson, 1979) to greater than 97%. This purified neurotoxin was coupled to Sepharose 2B which had been either "weakly" or "moderately" activated (Porath et al., 1973) by reaction with cyanogen bromide. Accordingly, after coupling with neurotoxin two degrees of Sepharose substitution were obtained. The capacity of the two affinity resins was 0.03–0.04 mg/mL and 0.35 mg of AcChR bound/mL, respectively.

The degree of substitution affected the amount of the adsorbed AcChR which could be specifically eluted by carbamoylcholine. When fetal calf AcChR was used, it was found that only 8% of the adsorbed AcChR could be eluted from the more highly substituted resin, while for the less substituted resin this value increased to 20–25% (see Table I).

Purification of AcChR. Torpedo californica AcChR purified by using the α-neurotoxin Sepharose 2B column with the higher degree of substitution had a specific activity of 8 nmol/mg of protein and showed in $NaDodSO_4$–polyacrylamide

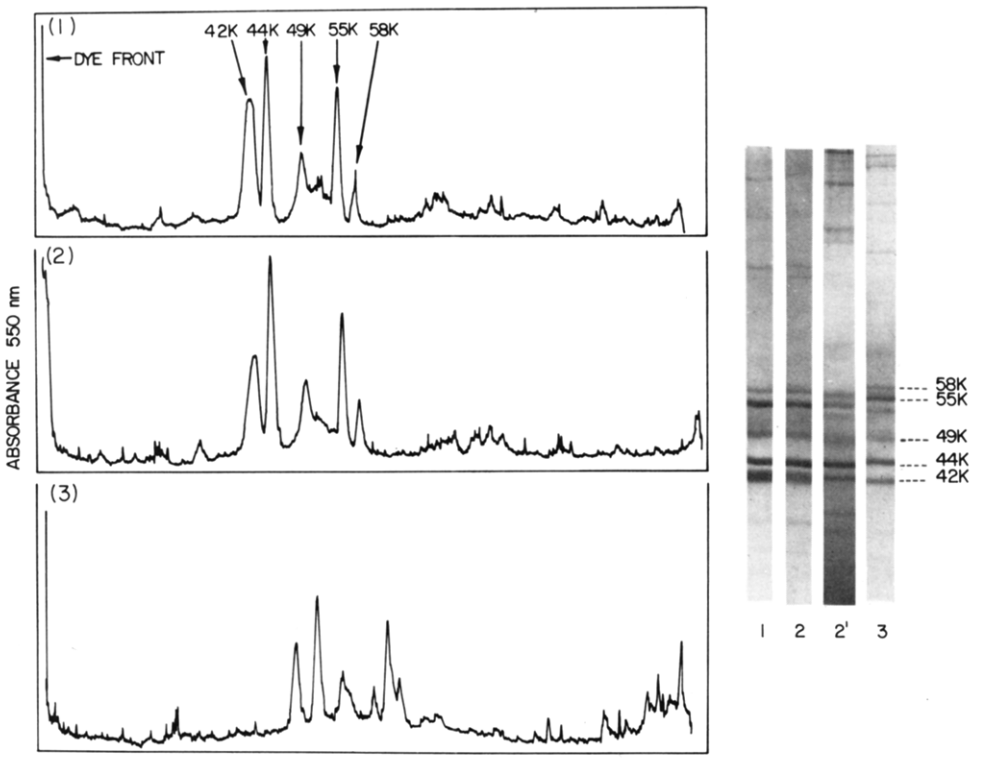

FIGURE 1: Polypeptide composition of fetal calf AcChR: polyacrylamide gels (8.75%) run in the presence of 0.1% $NaDodSO_4$. (Left) Scans of three representative preparations (1–3). (Right) Photographs of the corresponding gels (1–3). The gels labeled 2 and 2′ represent the carbamoylcholine- and $NaDodSO_4$-eluted AcChR from the same affinity resin. The molecular weight values for the various polypeptides are indicated.

Table I: AcChR Yield during Purification

	preparation		
	1	2	3
muscle weight (g)	1500	1500	1600
Triton extract[a] (nmol)	7.33	7.9	3.94
AcChR absorbed to affinity resin[a] (nmol)	4.57	3.85	2.59
carbamoylcholine eluate[a] (nmol)	0.725	0.972	0.483
total yield (%)	9.9	12.3	12.2

[a] Values are expressed as nmol of α-BuTx binding sites.

gel electrophoresis the usual pattern of four subunits with M_r 40K, 50K, 60K, and 65K, respectively [reviewed in Conti-Tronconi & Raftery (1982)].

For the purification of fetal calf AcChR, the affinity resin with the lower degree of substitution was used. A summary of the yield of AcChR at the major steps of the purification in three typical experiments is given in Table I. For each preparation frozen pellets equivalent either to 1500 g or to twice this amount of fetal muscle were used. The amount of AcChR obtained in the Triton X-100 extract ranged from 2.46 to 5.26 pmol/g of starting muscle, with an average of 3.94 ± 1.03 pmol/g. These numbers are low by 10–15% because about 10–15% of the Triton extract was lost when the lipid layer was aspirated. A fraction ranging between 49% and 66% of the AcChR contained in the Triton extract was adsorbed to the affinity resin. About 20% of the adsorbed AcChR was specifically eluted by incubation with Carb, giving an overall yield of 10–12%. When the affinity resin with the higher degree of substitution was used (capacity 0.35 mg of AcChR/mL of resin), only 8% of the adsorbed AcChR could be specifically eluted by Carb, and the overall yield was proportionally lower. After elution with Carb, the proteins still adsorbed to the column were recovered by incubation with 1.5% $NaDodSO_4$ (AcChR II).

Different preparations of AcChR eluted by Carb and dialyzed to remove the cholinergic ligand (AcChR I) had specific activities ranging between 4.6 and 6.8 nmol of α-BuTx binding sites/mg of protein with an average of 5.9 ± 0.8 nmol/mg (n = 4). The protein determination used for calculation of the specific activity was done by Lowry assay and/or amino acid analysis. The Lowry assay, in agreement with Martinez-Carrion et al. (1975), was found to yield values 1.08-fold higher of those obtained by amino acid analysis.

$NaDodSO_4$ Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was used both for analytical and preparative purposes and for concentrating the very diluted samples which were detached from the affinity column by incubation with $NaDodSO_4$ (AcChR II). So that this latter aim could be achieved, slab gels with a long stacking gel were used (as described under Methods). With such slabs, it was found that a test sample, having a volume of 2.5 mL/cm of slab width, and containing 3 μg/mL BSA, was concentrated into a sharp band. With this gel system it was possible to concentrate, separate, and study the polypeptides comprising AcChR II. Figure 1 reports the polypeptide composition of three typical fetal calf AcChR preparations. It clearly appears that the polypeptide pattern is highly reproducible in different preparations (Figure 1). Interestingly, the polypeptide composition of the eluate from the affinity column using $NaDodSO_4$ (AcChR II) was found to be almost identical with that of the carbamoylcholine eluate (AcChR I) (Figure 1, left, gels 2 and 2′), and it was therefore assumed to represent AcChR that was tightly bound to the resin. From the intensity of the Coomassie Blue staining, the amount of AcChR II obtained was estimated to represent at least twice as much protein as AcChR I, as expected considering the low efficiency of Carb elution (see Table I). All the preparations studied contained five polypeptides whose M_r ranged between 42K and 59K.

Table II: Composition of Purified AcChR

	fetal calf muscle AcChR (mol %)	*Torpedo* electroplax AcChR[a] (mol %)
Asp	8.85 ± 0.78	11.4
Thr	4.41 ± 0.58	6.2
Ser	7.37 ± 0.43	6.3
Glu	9.97 ± 1.32	10.3
Pro	6.88 ± 0.93	5.7
Gly	9.26 ± 0.94	4.9
Ala	5.6 ± 0.3	5.2
Cys	1.3 ± 0.3	1.3
Val	6.05 ± 0.56	7.5
Met	0.77 ± 0.33	1.9
Ilu	4.35 ± 0.25	7.6
Leu	7.97 ± 0.21	9.3
Tyr	2.97 ± 0.36	3.8
Phe	3.42 ± 0.27	5.0
His	2.23 ± 0.13	2.6
Lys	4.1 ± 0.45	5.7
Arg	4.05 ± 0.55	4.1
Glu-NH_2	1.61 ± 0.42	
Gal-NH_2	8.45 ± 4.03	

[a] Taken from Vandlen et al. (1979).

The molecular weight values ($\times 10^{-3}$) of these polypeptides for six different preparations were 58.3 (±0.47), 55.5 (±0.49), 49.3 (±0.47), 43.8 (±0.68), and 42.4 (±0.44). In addition to these peptides some preparations contained a band of M_r 41K, which by NH_2-terminal sequencing was demonstrated to be related to the AcChR subunits (unpublished results) and is likely to be a degradation product of one of the higher molecular weight subunits.

A sixth subunit of M_r 53 000 was consistently present in the preparations of AcChR II and only occasionally in AcChR I (Figure 1, arrows). In these latter preparations, when the 53K peptide was absent, a diffusely stained area was present in the area between the subunits of M_r 49K and 55K (Figure 1, arrows). The protein bands of M_r 42K, 44K, and 55K were consistently stained more intensely than the other ones. Faint bands of lower and higher molecular weight were erratically present, possibly because of partial degradation or cross-linking of AcChR subunits; in fact, they increased with aging of the preparation and with extended heating of the gel samples.

Amino Acid Composition. The amino acid composition of purified fetal calf AcChR is shown in Table II, where it is compared with the composition reported for *Torpedo californica* AcChR (Vandlen et al., 1979). The numbers represent the average composition of four different preparations of AcChR expressed as mole percent. For most amino acids, the values obtained are very close to those of *Torpedo* AcChR. The only amino acids which differed by more than 25% were Gly, Met, Ilu, and Phe. A large amount of amino sugars (2 mol % Glu-NH_2 and 8 mol % Gal-NH_2) was consistently found in the four preparations tested. The amount of Gal-NH_2 was variable from preparation to preparation, ranging from 3.9 to 14 mol %. The amino acid composition of the polypeptide of M_r 44K corresponded to that of actin (Elzinga et al., 1973).

Precipitation by Anti-actin Antibodies. Rabbit anti-actin serum consistently precipitated 10% of the purified fetal calf AcChR present in the assay mixture.

Physical Properties. Upon isoelectric focusing, purified fetal calf AcChR I/[^{125}I]-α-BuTx complexes consistently showed the presence of a species with pI = 5.16 ± 0.13 (n = 7) (Figure 2A). In most experiments, a second peak at pI = 6.05 ± 0.18 (n = 5) was present (Figure 2B). Under the same conditions,

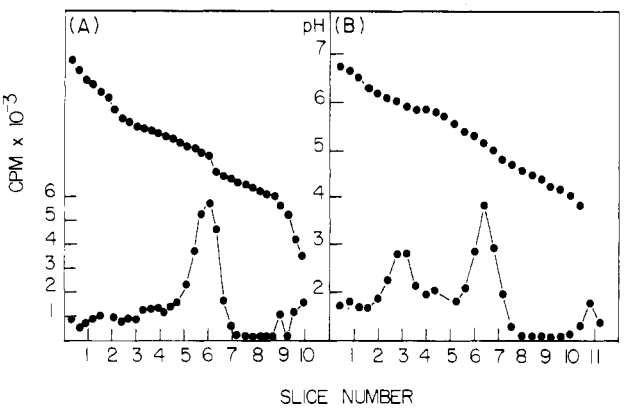

FIGURE 2: Isoelectric focusing of (A) purified *Torpedo californica* and (B) fetal calf AcChR as their [^{125}I]-α-BuTx complexes. *Torpedo* yielded an apparently single isoelectric species of pI = 5.4 ± 0.2 while two separate isoelectric forms were found for fetal calf AcChR.

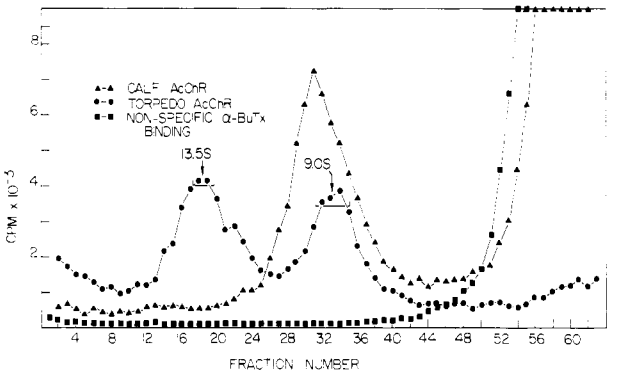

FIGURE 3: Sedimentation of AcChR in sucrose gradients. Linear density gradients (5–20% sucrose) were run by using samples of [^{125}I]-α-BuTx-labeled AcChR from *Torpedo californica* (●) or from fetal calf muscle (▲). In addition a blank of fetal calf AcChR preincubated with unlabeled α-BuTx prior to addition of [^{125}I]-α-BuTx was run as a control (■).

purified *Torpedo* AcChR/[^{125}I]-α-BuTx complexes showed a single charged species with pI = 5.0 ± 0.2 (n = 2). The results obtained for sedimentation of calf AcChR are reported in Figure 3.

Discussion

The method reported in this paper allows the purification of AcChR from mammalian muscle in relatively large amounts (in the nanomole range), sufficient for characterization of the chemical and physical properties not only of the AcChR complex but also of its isolated subunits, and to permit its use as an antigen in the study of the immune mechanism causing myasthenia gravis (Drachman, 1978; Conti-Tronconi et al., 1980). Under optimal conditions we obtained from 3000 g of fetal calf muscle about 250 μg of native AcChR, i.e., 1 nmol, and at least twice as much $NaDodSO_4$-denatured AcChR, suitable for the purification of subunits. The AcChR we isolated is likely to be in its intact, native form because it showed, upon $NaDodSO_4$–polyacrylamide gel electrophoresis, a complex subunit pattern, reminiscent of the pattern of intact *Torpedo* AcChR [reviewed in Conti-Tronconi & Raftery (1982)].

Several factors were critical for obtaining high yields of such intact AcChR: (1) the immediate homogenization of the dissected muscle in the presence of protease inhibitors, (2) the use of a highly purified α-neurotoxin as a ligand, thus reducing the possibility of coupling contaminants with proteolytic ac-

tivity of the resin, which would easily degrade the adsorbed AcChR during the relatively long times required for its adsorption to and desorption from the affinity resin, and (3) the use of an affinity resin with a low degree of substitution. Given the high stability of AcChR/toxin complexes, it is crucial to have a low local concentration of bound toxin for more efficient desorption of AcChR and a better overall yield. The use of an affinity resin with a 10-fold higher capacity (0.3 mg vs. 0.03 mg of adsorbed AcChR/mL of gel) reduced to less than half (from 20% to 8%) the fraction of adsorbed AcChR which could be specifically desorbed by Carb under our conditions.

The specific activity of our purified AcChR preparations was about 75% of the theoretical value calculated assuming that mammalian AcChR has the same α-BuTx binding sites/molecular weight ratio as *Torpedo* AcChR. This lower figure could be due to (1) the presence of high concentrations of IAA which, at 2 mM, was demonstrated to partially inactivate mammalian AcChR (Shorr et al., 1981), (2) possible incomplete dialysis of the massive amounts of Carb used for the elution of AcChR from the affinity resin, thus slowing the approach to equilibrium for formation of toxin–AcChR complex, and (3) the presence of contaminants [in fact, one of the major peptides present upon $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of M_r 44K was demonstrated to be actin (B. M. Conti-Tronconi, C. Gotti, M. W. Hunkapiller, and M. A. Raftery, unpublished results), which as discussed later is physically associated with at least part of the AcChR and copurifies with it].

Definitive identification of the subunits forming the AcChR complex will stem from their amino acid sequence if, like in *Torpedo* (Raftery et al., 1980) and *Electrophorus* (B. M. Conti-Tronconi, M. W. Hunkapiller, J. M. Lindstrom, and M. A. Raftery, unpublished results) AcChR, they are homologous peptides, as can be deduced from the frequently observed cross-reactivity of the anti-subunit antibodies in these species and in muscle (Claudio & Raftery, 1977, 1980; Lindstrom et al., 1979b, 1980; Tzartos & Lindstrom, 1980). However, the consistency of the polypeptide pattern we have found in muscle AcChR preparations strongly suggests that they all are part of the AcChR complex. It is not possible, so far, to decide whether some of them are degradation products of higher molecular weight subunits or the same subunit with different degrees of glycosylation. The subunit patterns of AcChR eluted with Carb or $NaDodSO_4$ were essentially identical, thus confirming that after extensive washing of the column the AcChR was the only protein which remained adsorbed. The only difference in the subunit pattern was the consistent presence of a peptide of M_r 53K in AcChR II, which was sometimes present in AcChR I. However, when this peptide was not clearly defined, a diffusely stained area was observed in this region, suggesting that the peptide was present but possibly modified by proteases during or after its concentration.

The different staining intensities of the bands suggest that their relative ratio could be different from that found for *Torpedo* AcChR (Lindstrom et al., 1979a,b; Raftery et al., 1980); i.e., the 42K subunit does not appear to be the prevalent one in mammalian AcChR. However, in the case of *Torpedo* it has been demonstrated that the efficiency of Comassie Blue staining is strongly dependent on the concentration of the protein applied to the gel (Strader et al., 1980), as well as on other factors such as the amino acid or sugar composition of the peptide. A more reliable method is needed for assessing the stoichiometry of these subunits, as was used for *Torpedo* (Strader et al., 1980).

The amino acid composition is similar to that of *Torpedo* AcChR (Vandlen et al., 1979). The presence of sugars was demonstrated in AcChR from both electric organ (Raftery et al., 1974; Meunier et al., 1974; Mattson & Heilbronn, 1975; Vandlen et al., 1979) and mammalian muscle (Shorr et al., 1978; Nathanson & Hall, 1979). Calf AcChR has a high content of amino sugars similar to that reported for *T. marmorata* AcChR (Mattson & Heilbronn, 1975). The variable amounts of Gal-NH_2 in different preparations (Table II) could be due to heterogeneity of the calf embryos used as the source of muscle since the animals differed in their degrees of maturation.

The fact that about 10% of the purified fetal calf AcChR could be precipitated by using specific anti-actin antibodies indicates a physical association of the AcChR and actin. As already discussed, a band of M_r 44 000 consistently copurified with the AcChR (in 15 preparations of Carb-eluted AcChR and in 16 preparations of $NaDodSO_4$-eluted AcChR). The molecular weight of this peptide (M_r 44 000) suggests its identity with actin, and this was proven by its amino acid composition and by the binding of anti-actin antibodies to the isolated peptide (B. M. Conti-Tronconi, C. Gotti, M. W. Hunkapiller, and M. A. Raftery, unpublished observation). In addition to its well-documented function in muscle contraction, actin has been shown to be a component of many types of nonmuscle cells [reviewed in Clark & Spudich (1978), Hitchcock (1979), and Lazarides (1980)] and is frequently associated with cell membranes, although the mechanism of association is not known (Tilney, 1976; Tilney & Mooseker, 1976; Spudich & Cooke, 1975). Actin is also present at the synapse in several species (Toh et al., 1976), where its function has not been determined. The AcChR is known to form stable patches both in the postsynaptic membrane and along the plasma membrane of denervated or noninnervated muscle and muscle cells [reviewed in Fambrough (1979) and Conti-Tronconi & Raftery (1982)]. Within these areas, the AcChR has very little rotational (Rousselot & Devaux, 1977; Lo et al., 1980) and lateral (Weinberg et al., 1981) mobility. The factors controlling this stability are still undefined. In addition to interactions between AcChR molecules, such as the formation of dimers of *Torpedo* AcChR, association with other extrinsic proteins could play a role. In *Torpedo* postsynaptic membranes, a protein of M_r 43K, distinct from actin (Strader et al., 1980), has been suggested to immobilize the AcChR, since its removal by alkali extraction causes a rearrangement of AcChR molecules (Barrantes et al., 1980) and an increase in their rotational mobility (Lo et al., 1980). No such protein has been identified so far for muscle AcChR preparations, and the demonstration that actin consistently copurifies with this AcChR and can be involved in a physical association with it suggests that actin is a possible candidate for this role.

Isoelectric focusing of purified fetal calf AcChR complexed to [^{125}I]-α-BuTx revealed the consistent presence of a charged species with pI = 5.16, which is close to that found for the AcChR complex from fish electric organ and from muscle of other mammalian species [reviewed in Conti-Tronconi & Raftery (1982)]. Since the pI of actin is in this range (Lazarides & Balzer, 1978) and at least 10% of the purified AcChR was physically associated with actin, it is possible that this second peak was due to the complex between [^{125}I]-α-BuTx and the AcChR molecules associated with actin. On the basis of our determination of the specific activity of the mammalian AcChR preparations (75% of theoretical) this slightly low value is due to the presence of 25% actin by weight and since only 10% of the AcChR seemed to be associated with

actin, these AcChR molecules should have an average of 20 molecules of associated actin. This large amount of actin could explain the large p*I* shift for this particular AcChR fraction.

The sedimentation coefficient of fetal calf AcChR is very similar to that of *Torpedo* AcChR monomers (Figure 3). No indication of the presence of a dimeric form as found in *Torpedo* was obtained. However, the dimers of *Torpedo californica* AcChR are extremely sensitive to the action of some proteases such as trypsin (Conti-Tronconi et al., 1982) and chymotrypsin (Huang, 1979). With trypsin, the majority of dimers can be split to monomers when the $NaDodSO_4$–polyacrylamide gel electrophoresis pattern is still apparently unaffected (Conti-Tronconi et al., 1982). Thus, the possibility that also calf AcChR can be present in the native state in a dimeric form, cleaved during the isolation procedure, cannot be excluded. Dimeric forms of muscle AcChR seem to occur in other species, like rat (Froehner et al., 1977) and cat (Barnard et al., 1978). Another possibility is that in calf muscle the dimers are held together by noncovalent interactions and they fell apart upon solubilization.

The sedimentation coefficient of 9.25 S suggests a molecular weight similar to that of *Torpedo* and *Electrophorus* AcChR monomers, i.e., $M_r \sim 250K$. This would fit with a subunit stoichiometry of the $\alpha_2\beta\delta\gamma$ type for the subunits of M_r 42K, 49K, 55K, and 58K (total M_r 246K). The relative intensity of these bands on $NaDodSO_4$ gel scans does not seem, however, to substantiate this idea. Thus, until reliable experimental data are available, any conclusion on the subunit stoichiometry of mammalian AcChR seems unwarranted.

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Physical Studies of Cell Surface and Cell Membrane Structure. Deuterium Nuclear Magnetic Resonance Studies of *N*-Palmitoylglucosylceramide (Cerebroside) Head Group Structure†

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ABSTRACT: Deuterium Fourier-transform nuclear magnetic resonance spectra of *N*-palmitoyl[2,3,4,6,6-$^2H_5$]glucosylceramide, *N*-palmitoyl[1-^2H]glucosylceramide, *N*-palmitoyl[5,6,6-$^2H_3$]glucosylceramide, and *N*-palmitoyl[6,6-$^2H_2$]glucosylceramide have been obtained at 55.3 MHz (corresponding to a magnetic field strength of 8.5 T) for lipids as multilamellar dispersions in excess water at 90 °C, above the gel to liquid-crystal phase transition temperature (T_c = 82 °C). Spectra were also obtained for these same lipids dispersed with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, and cholesterol, all in excess water at 90 °C. The results are analyzed in terms of a model in which the lipid undergoes fast axial diffusion, together with a "wobbling" of the polar head group, by mathematical methods similar to those used previously for the choline and ethanolamine head groups in phosphatidylcholines and phosphatidylethanolamines [Skarjune, R., & Oldfield, E. (1979) *Biochemistry 18*, 5903-5909]. However, contrary to the results obtained in the previous study, which indicated many possible conformations for the choline and ethanolamine head groups, results with labeled cerebrosides yield at most a few orientations for the glucose head group in each of the systems studied. Furthermore, where multiple solutions do occur, they fall within a narrow orientational subspace so that all solutions exhibit the same general features. We also show that the order parameter describing the head group wobble is fully determined for each system, and it indicates a rather mobile structure for the cerebroside head group, in a variety of environments. In each system studied, the polar head group projects essentially straight up from the bilayer surface into the aqueous region, thereby permitting maximum hydration of the four glucose hydroxyl groups by bulk water molecules.

Knowledge of the structure and dynamics of cell surface glycosphingolipids may be of considerable importance for understanding functional differences between normal and transformed cell membranes (Hakomori, 1973, 1975; Tooze, 1973; Clarkson & Baserga, 1974; Lee & Smith, 1974). To date, many studies have shown that these lipids undergo dramatic changes in composition upon malignant transformation (Hakomori & Murakami, 1968; Hildebrand et al., 1971, 1972; Seifert & Uhlenbruck, 1965; Kostic & Buchheit, 1970; Karlsson et al., 1974). These changes involve primarily a simplification of the (polar) carbohydrate residues (Tooze, 1973; Hakomori, 1970, 1971; Robbins & Macpherson, 1971; Sakiyama et al., 1972; Critchley & Macpherson, 1973; Kijimoto & Hakomori, 1972; Nigam et al., 1973). This suggests that important biological properties of the glycosphingolipids may be associated with the polar head group region of these molecules. Clearly then, it should be of considerable interest to examine the head groups of various glycosphingolipids by nuclear magnetic resonance (NMR)[1] spectroscopic and other physical methods in order to obtain an understanding of the static and dynamic structures of these species, an approach that might eventually provide valuable insights concerning cellular recognition processes at the molecular level.

In this paper we present the results of deuterium (^2H) NMR experiments with aqueous multilamellar dispersions of *N*-palmitoylglucosylceramide (PGLC) specifically labeled with deuterium in the carbohydrate ring. In contrast to work previously done with ^2H-labeled phosphatidylcholine and phosphatidylethanolamine, which yielded poorly defined results (Seelig et al., 1977; Seelig & Gally, 1976; Skarjune & Oldfield, 1979a), we show in this publication that all solutions for the glucose head group region of PGLC in a variety of environments show the same general orientational features. We use a very simple model of molecular motion in which the lipid molecules undergo fast axial diffusion, with the sugar moiety

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[1] Abbreviations: PGLC, *N*-palmitoylglucosylceramide; [2,3,4,6,6-$^2H_5$]PGLC, *N*-palmitoyl[2,3,4,6,6-$^2H_5$]glucosylceramide; [1-^2H]PGLC, *N*-palmitoyl[1-^2H]glucosylceramide; [5,6,6-$^2H_3$]PGLC, *N*-palmitoyl[5,6,6-$^2H_3$]glucosylceramide; [6,6-$^2H_2$]PGLC, *N*-palmitoyl[6,6-$^2H_2$]glucosylceramide; NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; SGADC, *N*-(2-hydroxystearoyl)galactosyldihydroceramide; PGAC, *N*-palmitoylgalactosylceramide.

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