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Immunochemical and Molecular Differentiation of 43 000 Molecular Weight Proteins Associated with *Torpedo* Neuroelectrocyte Synapses†

Reinhard Gysin, Beverly Yost, and Steven D. Flanagan*

ABSTRACT: Synaptic membranes, highly enriched in nicotinic receptor, contain three 43 000 molecular weight (M_r) peripheral proteins (distinctive in their peptide mapping profiles and earlier designated ν_1, ν_2, and ν_3) as well as the receptor $\alpha_2\beta\gamma\delta$ integral membrane subunits. Of the three proteins, only ν_1 is copurified with the membrane-bound receptor, while ν_2 and ν_3 are prominent cytosolic proteins, which are retained at significant levels in receptor-rich membranes during multistep centrifugation and affinity partitioning purification procedures [Gysin, R., Wirth, M., & Flanagan, S. D. (1981) *J. Biol. Chem. 256*, 11373–11376]. Peptide mapping analysis of *Torpedo* ν_3 and rabbit skeletal actin indicates that the two proteins are closely related. The enzymatic activity, creatine phosphokinase (EC 2.7.3.2), copurifies with ν_2 during chromatofocusing fractionation of the cytosol. The *Torpedo* electroplax form of creatine phosphokinase has an electrophoretic mobility identical with that of the mammalian skeletal muscle form of the enzyme. Upon release of the membrane-bound forms of ν_1, creatine phosphokinase, and actin by the action of mild alkali, ν_1 remains in a high molecular weight form. Dissociation of ν_1 into lower molecular weight species requires urea or sodium dodecyl sulfate ($NaDodSO_4$). Preparation of essentially pure ν_1 was achieved by eluting the ν_1 protein spots directly from $NaDodSO_4$–isoelectric focusing gels loaded with alkali extracts derived from membranes highly enriched in nicotinic receptor. Amino acid compositions of the purified fractions indicate that ν_1 and *Torpedo* creatine phosphokinase have distinct amino acid compositions from each other and from that of actin. In order to determine whether the observed differences in the peripheral protein peptide mapping and amino acid composition profiles are reflected as well in their antigenic properties, we have prepared an antiserum against an electrophoretically purified fraction highly enriched in ν_1. This antiserum is essentially monospecific for ν_1, detecting as little as 15 ng of ν_1 present in alkali extracts of affinity-purified membranes. The immunochemical analysis serves to emphasize the conclusion that, although ν_1 overlaps in both molecular weight and isoelectric focusing parameters with ν_2 (creatine phosphokinase) and ν_3 (*Torpedo* actin), they display no evolutionary interrelationships, having distinct antigenic sites, solubility properties, and amino acid compositions.

The subsynaptic region of the neuromuscular junction contains nicotinic acetylcholine receptor (nAChR)[1] molecules at such high densities (Fertuck & Salpeter, 1974) that there is little room within the plane of the membrane bilayer for macromolecular components other than the known $\alpha_2\beta\gamma\delta$ or core pentameric integral membrane subunits (Weill et al., 1974; Lindstrom et al., 1980a; Raftery et al., 1980). One explanation for this dense packing and restricted distribution of receptor is that the nAChR could form higher order polymers of the core pentameric integral membrane subunits via the formation of disulfide bonds between the δ-subunits (Chang & Bock, 1977; Hamilton et al., 1977, 1979). But dimers of the core pentameric structure ($\alpha_2\beta\gamma\delta$–S–S–$\delta\gamma\beta\alpha_2$) are the predominant species purified from *Torpedo*, and thus far, only the core pentameric structure itself has been purified from *Electrophorus* and mammalian muscle (Lindstrom et al., 1980b; Conti-Tronconi et al., 1982; Gotti et al., 1982; Einarson

† From the Membrane Neurochemistry Section, Division of Neurosciences, Beckman Research Institute of the City of Hope, Duarte, California 91010. *Received June 13, 1983.* Supported by National Science Foundation Grant BNS-8205917, by a grant from the Muscular Dystrophy Association, and by a supplementary grant from the California Chapter of the Myasthenia Gravis Foundation. This work was made possible in part by support from the Conrad Platt Research Fund.

[1] Abbreviations: BSA, bovine serum albumin; CPK, creatine phosphokinase (EC 2.7.3.2); HSS, high-speed supernatant; nAChR, nicotinic acetylcholine receptor; $NaDodSO_4$, sodium dodecyl sulfate; TBS, Tris-buffered saline; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)ammomethane; alkaline (pH 11) extracts were from purified *Torpedo* electroplax membranes.

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et al., 1982); therefore, higher order interactions of the nAChR within the plane of the membrane have yet to be explained. The above considerations suggest the possible involvement of other macromolecules in organizing the neuromuscular junction and the homologous *Torpedo* neuroelectrocyte synapse. Recently, several investigators have focused their attention upon peripheral membrane proteins that are copurified with nAChR in its native membrane environment (Sobel et al., 1978; Neubig et al., 1979; Moore et al., 1979; Elliot et al., 1980; Barrantes et al., 1980; Johansson et al., 1981; Gysin et al., 1981). These peripheral proteins were first observed as a band of 43 000 molecular weight upon $NaDodSO_4$–polyacrylamide gel electrophoresis (Sobel et al., 1977, 1978). Separation of the 43 000-dalton band using isoelectric focusing reveals more than seven discrete protein spots (Saitoh & Changeux, 1980; Gysin et al., 1981). These two-dimensional gel spots may be grouped into three classes designated ν_1, ν_2, and ν_3 based upon their peptide mapping profiles (Gysin et al., 1981). Two of these proteins, ν_1 and ν_2, overlap substantially in their isoelectric focusing and molecular weight parameters. Peptide mapping analysis tends to emphasize the differences between proteins so that even proteins that are closely homologous, based upon amino acid sequence analysis, can yield quite different peptide maps (Raftery et al., 1980; Froehner & Rafto, 1979). Thus, it was possible that the three distinct proteins observed were in fact evolutionarily related proteins produced by gene duplications and subsequent specialization. To evaluate this possibility and to extend our understanding of the molecular, immunochemical, and biochemical properties of the ν proteins, we have purified them to homogeneity, compared their amino acid compositions, evaluated their immunological cross-reactivity, and compared the individual ν proteins with known proteins of similar electrophoretic mobilities (including actin and CPK). The results of our studies suggest that ν_1 is a unique nonreceptor structural component present at synapses in the *Torpedo* electroplax.

Experimental Procedures

Preparation of Membranes, Alkaline Extracts, and Cytosol. Membranes from *Torpedo californica* electroplax, highly enriched in nicotinic acetylcholine receptor, were prepared by sucrose density gradient centrifugation and affinity partitioning as previously described (Johansson et al., 1981; Gysin et al., 1981). The cytosolic fraction used for purification of ν_2 was the supernatant (HSS) of the first high-speed centrifugation (75 min at 149000*g*), after homogenization in 1 M NaCl (Reed et al., 1975; Johansson et al., 1981). Peripheral membrane proteins were obtained from affinity-purified membranes by alkaline extraction using the method of Neubig et al. (1979).

Gel Electrophoresis and Peptide Mapping. $NaDodSO_4$ gel electrophoresis was performed on 10% polyacrylamide gels using the system of Laemmli (1970). Standard proteins used for molecular weight determination were myosin heavy chain (M_r 200 000), β-galactosidase (M_r 116 000), phosphorylase *a* (M_r 94 000), BSA (M_r 68 000), catalase (M_r 60 000), ovalbumin (M_r 45 000), aldolase (M_r 40 000), carbonic anhydrase (M_r 30 000), and soybean trypsin inhibitor (M_r 21 500). Two-dimensional gel electrophoresis was performed according to O'Farrell (1975), with the modifications previously described (Gysin et al., 1981). Tryptic peptide maps were obtained as previously described (Elder et al., 1977; Gysin et al., 1981).

Chromatofocusing. Fractionation of *Torpedo* cytosol (HSS fraction) was performed on a chromatofocusing column with PBE 94 (Polybuffer exchanger, Pharmacia) equilibrated in 0.025 M Tris-HCl, pH 8.3. The sample was dialyzed against the same buffer, and as much as 100 mg of protein in 50 mL was applied to the 20-mL bed volume. A three-step protocol was used with elution buffers according to manufacturer's instructions designed for a gradient of pH 8–4. For reproducible pH gradients and quantitative protein recovery, we found it necessary to use HCl for buffer adjustment rather than acetic acid as recommended by Pharmacia. The following three elution buffers were used sequentially: (I) 240 mL of 10% PB 96 (Polybuffer, Pharmacia), pH 6.5; (II) 150 mL of 14.7% PB 74, pH 5.0; and (III) 200 mL of 14.7% PB 74, pH 3.8. Fractions of 5 mL were collected at 14–23 mL/h and pooled according to the protein profile measured by the absorbance at 280 nm. The pooled fractions were concentrated and the Polybuffer components removed by repeated filtration through an Amicon YM-30 membrane.

Creatine Phosphokinase Assay and Protein Determination. Creatine phosphokinase activity (EC 2.7.3.2) was measured spectrophotometrically by monitoring NADPH production in a reaction coupled with hexokinase and glucose-6-phosphate dehydrogenase, using creatine phosphate as substrate (Forster et al., 1974). The specific myokinase inhibitor P^1,P^5-diadenosine pentaphosphate (Sigma Chemical Co.) was included in the reaction mixture as described by Munson et al. (1982). The isoenzyme pattern was determined fluorometrically after electrophoresis on thin-layer agarose gels (Corning Medical) according to the instructions provided by Corning. Protein determinations were made by using the Coomassie Blue binding procedure (Bio-Rad Laboratories) with BSA as a standard.

Preparation of Antisera. Antisera were prepared in rabbits by injection of electrophoretically purified M_r 43 000 proteins from alkaline extract[1] (sera 1 and 2) or the undenatured alkaline extract from affinity-purified membranes (sera 3 and 4). The 43 000-dalton band was cut out from preparative $NaDodSO_4$ gels of alkaline extract after visualization by staining for 1–2 min with Coomassie blue (0.125% in 50% methanol and 7.5% acetic acid) and rinsing of the gel in H_2O. The protein was electroeluted from the gel into a dialysis bag attached to the bottom of a glass tube fitted with a porous disk (Bio-Rad Econo column, 10 cm long, 0.7 cm id). The tube was inserted into a tube gel apparatus with both buffer chambers filled with 25 mM Tris-HCl, pH 8.5, and 0.1% $NaDodSO_4$. Electroelution was carried out at 150 V until all stain had reached the bottom of the dialysis bag. Each rabbit was injected subcutaneously with 0.2–0.4 mg of electroeluted M_r 43 000 protein or with 0.5–1.0 mg of alkaline extract in 1 mL of saline homogenized with 1 mL of Freund's adjuvant. An initial injection in complete adjuvant was followed by two to three booster injections in incomplete adjuvant at intervals of 2–4 weeks. Serum was prepared from bleedings obtained 5–10 days after the last injection.

Immunoprecipitation of ^{125}I-Labeled Alkaline Extract Components. Alkaline extract was radioiodinated with the Chloramine-T method. Immunoprecipitation was carried out in a buffer containing 50 mM Veronal sodium, pH 8.2, 80 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3, 0.5% Triton X-100, 0.1% $NaDodSO_4$, and 5 mg/mL BSA. Samples, containing 0.4 mL of ^{125}I-labeled alkaline extract and 0.1 mL of antiserum (diluted 1:10), were incubated for 30 min at room temperature, followed by addition of 0.1 mL of a 10% suspension of protein A bearing *Staphylococcus aureus* (Pansorbin, Calbiochem). The incubation was continued for 30 min, and the samples were stored at 4 °C overnight. The protein A bound anti-

gen–antibody complexes were centrifuged in a Beckman microfuge. The pellets were washed by resuspending once in 0.5 mL of precipitation buffer and twice in 0.5 mL of buffer without BSA.

Immunoblotting. Replicas of electrophoretic patterns were prepared by transfer onto 15 × 9.2 cm nitrocellulose filter sheets (Bio-Rad) using the method of Towbin et al. (1979). The blots were washed and incubated with antiserum essentially as described by Matus et al. (1980), using a buffer containing 50 mM Tris, pH 7.4, and 150 mM NaCl (TBS). The blots were first incubated for 40 min at 40 °C in a shaking water bath with 33 mL of 3% BSA in TBS. After being rinsed briefly with TBS, the blots were placed in Parafilm boats, overlayed with 10 mL of antiserum, diluted 1:50 in TBS, and incubated for 90 min at room temperature with shaking. After being washed 4 times for 40 min with 100 mL of TBS, the blots were placed in new Parafilm boats and subsequently incubated for 90 min in 8 mL of TBS containing (2–4) × 10^7 cpm of ^{125}I-labeled protein A (radioiodinated by using Chloramine-T to 12–20 μCi/μg) and 0.1% BSA. The blots were washed for 3 h with six changes of 100 mL of TBS, or with three changes overnight, and air-dried. Autoradiography was then performed by exposing the blots to Kodak XAR-5 X-ray film.

Amino Acid Analysis. For determination of the amino acid composition, the ν_2 (CPK) fraction from the chromatofocusing column was reduced by boiling for 2 min in dissociation buffer and subsequently alkylated with iodoacetamide (Lane, 1978). After $NaDodSO_4$ electrophoresis, an adjacent lane was stained with Coomassie Blue, and the purified protein was eluted from the unstained region of the gel. The same procedure was used for elution as described above, with 10 mM NH_4HCO_3 substituted as the elution buffer. Pure ν_1 was obtained by elution from two-dimensional gels of alkaline extract. Prior to electrophoresis, the sample was reduced for 10 min at 50 °C in dissociation buffer and alkylated. The proteins were precipitated with trichloroacetic acid added to a final concentration of 15% and collected by centrifugation for 30 min at 45 000 rpm in an SW 50.1 rotor. The tube walls were wiped with cotton swabs moistened with 10^{-3} M NaOH in order to remove excess acid. The sample was solubilized by using the standard procedure (Gysin et al., 1981), with the addition of 0.001% bromophenol blue to the solubilization mixture. Small increments of NaOH were then added until the indicator color had changed to dark blue. Control experiments had shown that the two-dimensional pattern of the ν proteins was not affected by this procedure. The spots containing ν_{1a} and ν_{1b} were cut out from the gels after visualization for 5 min in a solution containing 0.04% Amido Black in 50% methanol and 8% acetic acid and electroeluted.

The electroeluted purified protein samples were extensively dialyzed against 10 mM NH_4HCO_3 and lyophilized. Amino acid determinations were made from acid hydrolysates (48 h at 110 °C) containing 1–5 μg of protein by using the procedure of Del Valle & Shively (1979). Blank gels were run with sample buffer only, and the appropriate gel area was cut out and electroeluted. Amino acid data were corrected by subtracting the values obtained from these blanks (Brown & Howard, 1980).

Results

Peptide Mapping Analysis of Torpedo ν_3 and Rabbit Skeletal Muscle Actin Reveals Close Sequence Homology. *Torpedo* actin reportedly (Strader et al., 1980; Sobel et al., 1977) migrates more slowly than the receptor-associated 43 000-dalton band in the Laemmli $NaDodSO_4$ gel system that we have routinely employed for one-dimensional gel electrophoresis and as the second dimension of our two-dimensional electrophoresis (O'Farrell, 1975; Gysin et al., 1981). Since ν_3 migrated in the second (molecular weight) stage of the two-dimensional gel with a relative mobility virtually identical with that of ν_1 and ν_2, we had refrained from identifying ν_3 as actin. While parallel electrophoresis of skeletal muscle actin and alkaline extract samples in one-dimensional gels confirmed the observation of Strader et al. (1980), two-dimensional gel electrophoresis of actin and ν_3 reveals that they possess identical p*I* and molecular weight parameters, yielding an apparently lower molecular weight for *Torpedo* actin in the two-dimensional system than in the equivalent one-dimensional system (not shown). The identical electrophoretic properties of ν_3 and skeletal muscle actin suggested possible sequence homology, which was confirmed by peptide mapping analysis as displayed in Figure 1. Of the 12 major spots visible on autoradiographs of radioiodinated tryptic peptides, 10 are superimposable, while 2 peptides have different mobilities. This indicates extensive sequence homology between the two proteins and leads us to conclude that ν_3 is *Torpedo* actin, or a protein very closely related to actin. Our previous studies illustrated the marked differences in the peptide mapping profiles of ν_1, ν_2, and ν_3 (*Torpedo* actin), which were confirmed in a new series of peptide maps performed during the current study (not shown).

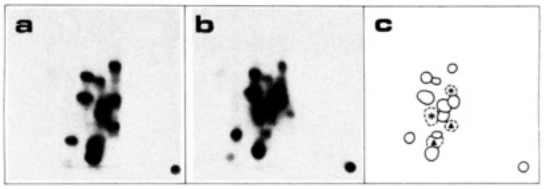

FIGURE 1: Comparison of peptide maps of actin and ν_3. Spots from two-dimensional gels were cut out and tryptic peptide maps obtained as described (Gysin et al., 1981; Elder et al., 1979). The peptide map (a) from rabbit skeletal muscle actin (Sigma) shows 12 major spots after 24-h exposure. The same number of spots is seen in (b), which was obtained from ν_3 originating from *Torpedo* cytosol. The spot in the bottom right-hand corner marks the sample origin. The diagram in (c) shows the position of 10 superimposable peptides (solid lines) and two peptides migrating at a different position (broken lines) for either protein. The peptide positions singular to the map of rabbit muscle actin are indicated with a triangle; an asterisk indicates the peptides unique to ν_3.

Purification of ν_2 to Homogeneity by Chromatofocusing. The HSS fraction from *Torpedo* electroplax is very rich in ν_2. In fact, ν_2 is the most prominent protein on two-dimensional gels in this otherwise very complex fraction (Gysin et al., 1981). Densitometric scans of $NaDodSO_4$ gels stained with Coomassie Blue indicated that the 43 000-dalton band contained ν_2 and *Torpedo* actin at levels ranging from 8 to 27% of the total soluble proteins among soluble protein samples examined. As visualized on two-dimensional gels, ν_2 usually represents more than half of the total M_r 43 000 band. As described below, gel filtration of HSS on AcA 34 indicated that ν_2 was eluted in a broad peak, designated fraction II, between the marker proteins ovalbumin (M_r 45 000) and aldolase (M_r 157 000). Therefore, under the high-salt conditions used for elution, ν_2 could be present in monomeric to tetrameric forms.

Two-dimensional gels of HSS reveal that ν_2 is more basic than most other soluble extroplax proteins, focusing in multiple spots between p*I* 6.8 and 7.2 (Gysin et al., 1981). It was therefore reasonable to assume that a purification method separating on the basis of charge or isoelectric point would prove useful. While conventional ion-exchange chromatog-

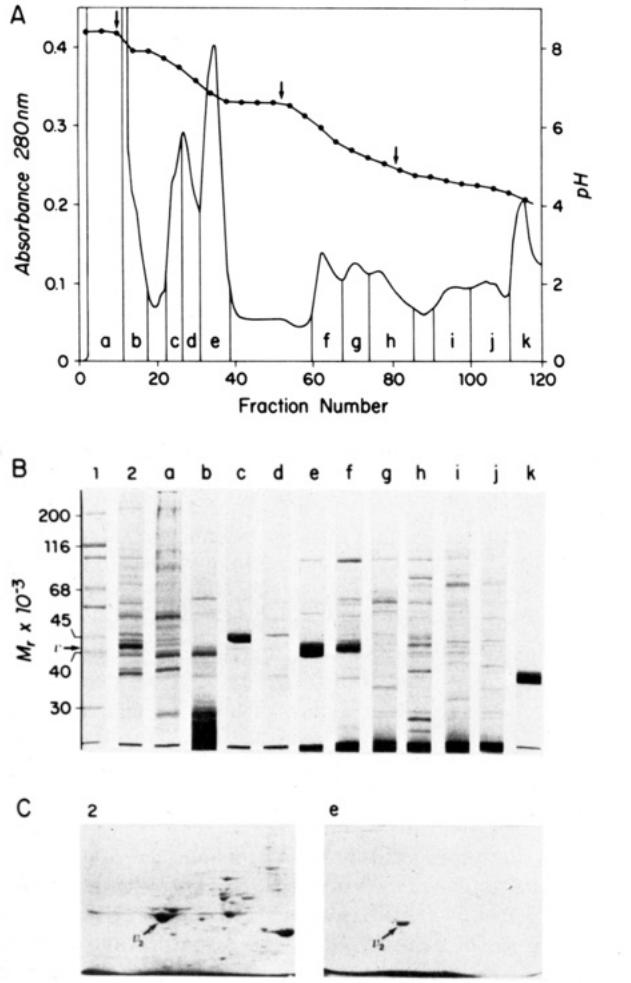

FIGURE 2: Chromatofocusing of *Torpedo* cytoplasm. *Torpedo* cytoplasm (HSS, 94 mg) was fractionated on a chromatofocusing column (Pharmacia) of 20-mL bed volume. A three-step elution protocol was used to generate a gradient between pH 8.3 and pH 4; the start of each buffer is indicated by the arrows. Fractions were pooled as indicated in panel A and analyzed by gel electrophoresis. The lanes in panel B show standard proteins (1), unfractionated HSS (2), and the pooled chromatofocusing fractions (a–k). The two-dimensional gels of HSS (panel C, 2) and pooled fraction e (panel C, e) illustrate the purification of ν_2 with this procedure. The amino acid composition of ν_2 (CPK) was determined after electroelution of the band from a preparative gel similar to the one shown in panel B, lane e.

raphy on DEAE-agarose (DEAE-Bio-Gel A, Bio-Rad) yielded some purification, the recovery of ν_2 was unsatisfactory. In contrast, a highly purified preparation of ν_2 was obtained with the chromatofocusing technique. This method is similar to isoelectric focusing insofar as proteins are separated on the basis of their isoelectric point (Sluyterman, 1981), but due to the concentrating effect of this method, the chromatofocusing column has a significantly higher capacity than earlier isoelectric focusing technology. In a typical run, 30 mL of HSS (94 mg of protein) was applied on a column of 20-mL bed volume (Polybuffer exchanger PBE 94, Pharmacia) in 0.025 M Tris-HCl, pH 8.3 (Figure 2). A three-step protocol was used as described under Experimental Procedures with buffers designed for a gradient of pH 8–4. We found it necessary for reproducible pH gradients and quantitative protein recovery to use HCl for the buffer adjustment rather than acetic acid as recommended by Pharmacia. Under these conditions, ν_2 was eluted in a fraction between pH 6.6 and 6.9, containing only minor amounts of contaminating proteins. After concentration and dialysis to remove the chromatofocusing buffer components, this fraction contained 9.4 mg of protein (10.2% of loading) and was estimated to be 85% pure in ν_2. The remaining impurities could be removed by preparative gel electrophoresis.

Table I: Copurification of ν_2 and Creatine Phosphokinase Activity on the Chromatofocusing Column[a]

	Torpedo HSS	ν_2 fraction
activity (units)	4210	3509
sp act. (units/mg of protein)	46.8	375
activity recovered (%)	100	83

[a] Data are from chromatofocusing experiments similar to the one in Figure 2 with 50 mL of HSS containing 90 mg of protein.

Identification of ν_2 as Creatine Phosphokinase. An approach to determining the functional roles of the ν proteins is to compare their molecular and functional properties with those of other known proteins. As described above, the identification of ν_3 as a form of *Torpedo* actin was based upon a comparison of its peptide mapping pattern with mammalian skeletal muscle actin. The identification of ν_2 as creatine phosphokinase (CPK; EC 2.7.3.2) is based upon different evidence, viz., the copurification of ν_2 with the enzyme activity during chromatofocusing. CPK subunits are generally between M_r 39 000 and 43 000 from a variety of species [see references in Watts (1973)], with the p*I* depending upon the isoenzyme type. The enzyme activity is especially rich in muscle tissue, and so it was not surprising to find the relatively high specific activity of 46.8 units/mg of protein in the cytosolic fraction of *Torpedo* electric organ (see Table I). During chromatofocusing, an 8-fold enrichment of CPK activity was observed with none of the enzyme activity localized outside the peak containing ν_2. The specific activity reported here is somewhat lower than the 480–720 units/mg reported for the purified isoenzymes from chicken and rabbit muscle (Eppenberger et al., 1967). The lower specific activity observed by us is likely to be due to the fact that we utilized frozen electric organ as a starting source for our studies, which is known to result in loss of CPK activity, especially in the absence of reducing agents. As can be seen from the gel of a similar fraction displayed in lane e of Figure 2, only very minor bands are present besides ν_2, consistent with our estimate of 85% purity. On the basis of the measured specific activity of this fraction, we conclude that the CPK enzyme activity is associated with ν_2 and not with one of the minor bands. Electrophoretic analysis of the isoenzyme pattern showed that all of the CPK activity comigrated with the muscle (MM) form of human CPK (data not shown). No activity was found comigrating with the brain-sepecific (BB) or mixed (MB) isozymes.

Amino Acid Composition of ν_1 and Torpedo CPK. Because the molecular weight and p*I* parameters of ν_1 and *Torpedo* CPK are so similar, it could be argued that a close relationship may exist between the two proteins that was not detected by using the very sensitive peptide mapping techniques used so far. It is known that only minor differences in primary structure can profoundly affect the immunological domains as well as the pattern of proteolytic peptide fragments. The overall amino acid composition was therefore determined, which allows us to compare a parameter that is less subject to evolutionary change. For this purpose, minor contaminating proteins still present after chromatofocusing were removed from CPK by $NaDodSO_4$ electrophoresis, and the pure protein was collected from the gel by electroelution. Pure ν_1 was obtained in quantities sufficient for amino acid analysis by elution from two-dimensional gels of alkaline extract. Only ν_{1a} and ν_{1b}, the more basic variants that are free from possible

Table II: Amino Acid Composition of ν_1 and *Torpedo* CPK[a]

amino acid	ν_1 (mol %)	CPK (mol %)
His	2.93	4.23
Lys	5.89	8.01
Trp	*b*	*b*
Arg	5.36	5.44
Cys	3.53[c]	1.29[c]
Asx	7.42	14.77
Thr	2.56	3.54
Ser	6.77	4.70
Glx	15.21	11.57
Pro	1.95	3.98
Gly	10.44	10.61
Ala	10.09	4.29
Val	3.65	5.61
Met	2.42	2.44
Ile	2.47	3.67
Leu	12.85	9.58
Tyr	3.46	2.30
Phe	2.30	3.97

[a] Determined from 1–5 μg of purified protein electroeluted after two-dimensional or $NaDodSO_4$ gel electrophoresis as described under Experimental Procedures. The values were corrected for amino acid contamination introduced by gel electrophoresis and other purification procedures by subtracting the blank values obtained from electroelution of the appropriate region of gels loaded with the corresponding sample blank. The values are averages of duplicate (CPK) or triplicate (ν_1) determinations, respectively. [b] Present; not corrected for destruction during hydrolysis. [c] Determined as carboxymethylcysteine.

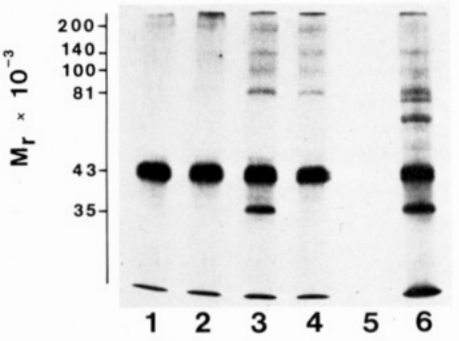

FIGURE 3: Specificity of antisera prepared against *Torpedo* M_r 43000 proteins or alkali extracts. Antisera from the four rabbits were tested for precipitating activity against alkaline extract radioiodinated by using the Chloramine-T method. ^{125}I-Labeled alkaline extract (9.5×10^5 cpm; specific activity 7.3×10^9 cpm/mg of protein) was incubated at a serum dilution of 1:50 and immunoprecipitated with *Staphylococcus aureus* (100 μL) as described under Experimental Procedures. For $NaDodSO_4$ gel electrophoresis, the protein A bound complexes were released from the *S. aureus* pellets by boiling 2 min in sample buffer. Lanes 1 and 2 are autoradiographs of immunoprecipitates obtained with antisera 1 and 2 (from rabbits immunized against the 43000-dalton band from Laemmli gels of alkali extract), which respectively precipitated 24% and 26% of the ^{125}I-labeled alkaline extract. Sera 3 and 4 (lanes 3 and 4), precipitating 35% and 31% of the ^{125}I-labeled alkaline extract, were obtained by immunizing against unfractionated alkali extract. Preimmune serum (lane 5) precipitated 2% of the radioactivity under the same conditions. The total ^{125}I-labeled alkaline extract before precipitation is shown in lane 6.

contamination with CPK, were used for the elution. The amino acid composition was corrected by subtracting the blank value obtained from the elution of the appropriate region from gels loaded with sample buffer only. This procedure has been shown to give reliable values despite the contamination that is introduced by gel material and dialysis tubing (Brown & Howard, 1980). The amino acid composition of ν_1 is substantially different from that of CPK (Table II), which in turn is similar to published amino acid compositions from CPK from several species [see references in Watts (1973)]. Also, no similarities can be found between ν_1 and the amino acid composition of muscular and cytoplasmic actin variants (Collins & Elzinga, 1975; Vandekerckhove & Weber, 1978).

Torpedo Actin and CPK Are Immunologically Distinct from ν_1. Two separate schemes were utilized to prepare antisera against the 43000-dalton proteins: In one protocol, we immunized two rabbits with alkaline extract prepared from membranes highly enriched in nAChR (Gysin et al., 1981). Preparation of the alkaline extract and the immunization protocol may be compared to that described by Froehner et al. (1981). Two additional rabbits were immunized with an electrophoretically purified preparation enriched in the 43000-dalton polypeptides present in the alkali extracts as described under Experimental Procedures. On the basis of two-dimensional electrophoresis, the preparation contained 70% ν_1 and 30% CPK and actin combined.

The four antisera were characterized by their ability to mediate the precipitation of protein components from an ^{125}I-labeled alkaline extract of membranes enriched in nAChR. Electrophoretic analysis of the immunoprecipitates obtained with each of the antisera served to highlight their unique binding profiles (Figure 3). Differences in the specific binding profiles are especially apparent when comparisons are made between the pair of antisera produced against the alkaline extract of the affinity-purified membranes and the pair produced against electrophoretically purified 43000-dalton proteins derived from the alkaline extract. The antisera prepared against the alkaline extract bind the relatively minor constituents of M_r 47000, 81000, 100000, 135000, 140000, and 190000 (Figure 3, lanes 3 and 4), while the antisera prepared against the 43000-dalton band exclusively precipitate proteins that migrate to that region of the gel (Figure 3, lanes 1 and 2). Differences are also apparent between individual antisera within a pair. For example, antiserum 3 is unique in its precipitating activity for a 35000-dalton protein frequently present in the alkaline extract, whereas antiserum 4 binds little of the 35000-dalton species. Antisera 1 and 2, prepared against the 43000-dalton $NaDodSO_4$ band from the purified alkaline extract, proved less effective as an Ouchterlony precipitating reagent than the antisera prepared against undenatured alkaline extract. The resulting precipitin lines were fainter and were best visualized by Coomassie Blue staining (data not shown). Nonetheless, the two antisera were highly effective in adsorption of the 43000-dalton components of the alkaline extract (Figure 3). In experiments not shown, two-dimensional gel electrophoresis of the immunoprecipitated ^{125}I-labeled alkaline extract indicates that antiserum 2 binds CPK as well as ν_1, whereas antiserum 1 binds exclusively ν_1. Thus, by immunoprecipitation criteria, antiserum 1 is essentially monospecific for ν_1.

The above results were confirmed by immunoblotting analysis of *Torpedo* electroplax samples, both derived from alkaline extract of purified membranes and crude homogenates (Figure 4). Antiserum 1 binds exclusively to the 43000-dalton band, in both alkaline extract and crude electroplax homogenates, while antiserum 4 stains a variety of molecular weight protein species. As described in detail below, the results of one-dimensional analysis were extended to two-dimensional gels (Figure 7), indicating that antiserum 1 detects exclusively ν_1.

Detection of ν_1 *in the 1 M NaCl Soluble Fraction from Torpedo Homogenates by a Monospecific Antiserum.* Because antiserum 1 binds ν_1 exclusively and is capable of detecting as little as 15 ng of antigen, it is useful for identifying ν_1 in subcellular fractions, even where it is present in very small

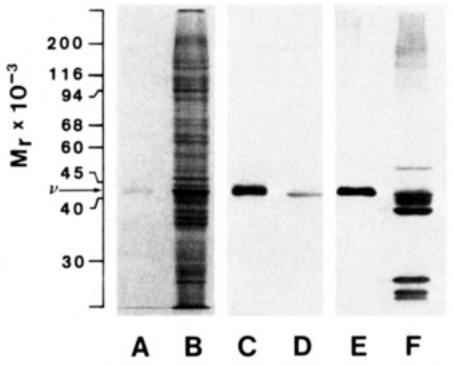

FIGURE 4: Immunoblot reaction of antisera 1 and 4 with alkaline extract and *Torpedo* homogenate. Alkaline extract (5 μg, lanes A, C, and E) and unfractionated homogenate from *Torpedo* electroplax (40 μg, lanes B, D, and F) were separated by $NaDodSO_4$ gel electrophoresis and stained with Coomassie Blue (A, B) or transferred to nitrocellulose paper (C–F). Subsequent incubation of the transfers (blots) with antiserum and visualization with ^{125}I-labeled protein A were carried out as described under Experimental Procedures. Antiserum 1 (lanes C and D) and antiserum 4 (lanes D and F) were used at a dilution of 1:50. Exposure time of the radioautographs was 40 min.

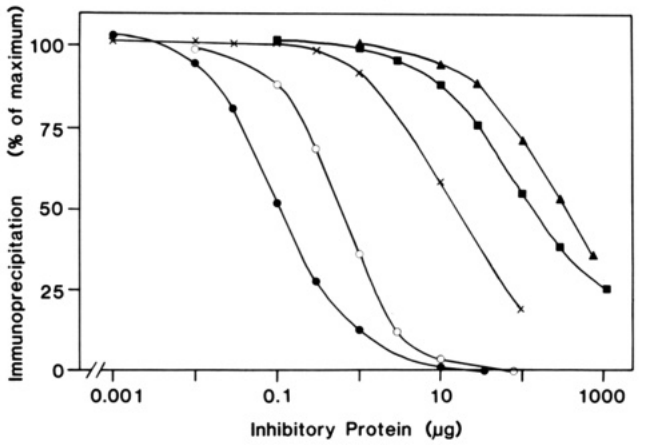

FIGURE 5: Immunoprecipitation of ^{125}I-labeled alkaline extract with antiserum 1: inhibition by membrane- and cytosol-derived fractions. Serum 1 (0.1 mL of 1:100 dilution) was preincubated for 30 min at 37 °C with the indicated amount of inhibitory protein in a volume of 0.45 mL. ^{125}I-Labeled alkaline extract was then added (2.6×10^5 cpm in 0.05 mL) and the incubation continued for 30 min at room temperature at a final serum dilution of 1:500. The immunoprecipitation with *S. aureus* (50 μL) was then carried out as described under Experimental Procedures. Under these conditions, 19.7% of the added radioactivity is precipitated specifically in the absence of inhibitor. All indicated values have been corrected by subtracting the amount of nonspecific precipitation, which is 1.2% of the total radioactivity added. The extent of nonspecifically bound ^{125}I-labeled alkaline extract was identical whether preimmune serum was added or not. The percentage of remaining immunoprecipitating activity is indicated for the membrane-derived alkaline extract (●), affinity-purified membranes (○), HSS (■), and the HSS-derived fractions I (×) and II (▲). (See Figure 6 for definition of HSS fractions.)

amounts. Quantitative assay of the ν_1 content of a given subcellular fraction is achieved by preincubation with antiserum 1 prior to addition of ^{125}I-labeled alkaline extract. Antibody-mediated precipitation of ^{125}I-labeled alkaline extract to protein A coated bacteria was used to determine the extent of the inhibitory activity (Figure 5). When applied to the measurement of the relative potencies of various *Torpedo* fractions, the dynamic range of the assay spanned over 5 orders of magnitude in protein concentration. For all subcellular fractions, the relative inhibitory potency of the various fractions correlated rather well with the expected order based upon assessments of the ν_1 content by Coomassie staining of two-

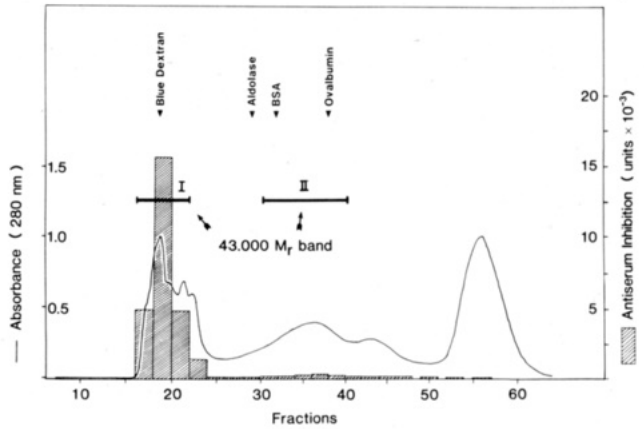

FIGURE 6: Fractionation of HSS by gel filtration. High-speed supernatant (HSS) from homogenized *Torpedo* electric organ was applied on an Ultrogel AcA-34 column (LKB, fractionation range 350 000–20 000 daltons, dimension of 48.5 cm × 2.5 cm) equilibrated in 1 M NaCl, 25 mM Tris-HCl, pH 8.3, and 0.01% NaN_3. Fractions of 5 mL were collected at a flow rate of 23 mL/h. For gels and inhibition assays, fractions were dialyzed against 10 mM NH_4HCO_3 and lyophilized. One-dimensional electrophoresis indicated that the 43 000-dalton proteins eluted within two broad peaks, as marked by horizontal bars. The first peak (I, fractions 17–22) coincided with the void volume of the column, whereas the second peak (II, fractions 31–40) eluted between M_r 40 000 and 140 000, according to standard proteins. Serum inhibition in the fractions was measured by preincubation of 50 μg of protein with antiserum 1 (1:100 dilution). Determination of the remaining precipitation activity was tested with ^{125}I-labeled alkaline extract as described for Figure 5. One unit of inhibition has been defined as the inhibiting activity achieved with 1 μg of protein from unfractionated HSS.

dimensional gels. Thus, alkaline extract was the most potent fraction, with 0.11 μg of protein required for 50% inhibition; 5 times higher levels of affinity-purified nAChR-enriched membrane protein (0.58 μg) were required for an equivalent inhibition, consistent with the measured content of ν_1 in the nAChR-enriched fractions (Johansson et al., 1981; Gysin et al., 1981). Unexpectedly, 130 μg of the HSS fraction also inhibited 50% of the ^{125}I-labeled alkaline extract binding. Coomassie Blue staining of two-dimensional gels of the membranous and cytosolic fractions had indicated that ν_1 was present exclusively as the membrane-bound species, with no detectable ν_1 in the HSS fraction, where considerable CPK and actin are observed. This apparent discrepancy was resolved by calculating the amount of ν_1 present in the HSS fraction. The estimated ν_1 content of the alkaline extract is 70% of the protein. An extrapolation of this value, using the amount required for 50% antiserum inhibition, indicates that the content of ν_1 is the HSS fraction is 0.06% of the protein, below that detectable by Coomassie Blue staining.

The immunoblotting technique permitted an identification of the inhibiting species by determination of their electrophoretic properties. For example, we observed low levels of antibody binding components whose electrophoretic mobilities were identical with ν_1 in the HSS (not shown). That antiserum 1 indeed does not cross-react with CPK, actin, or other proteins was confirmed by the combination of gel filtration (Figure 6) followed by immunoblotting of fraction II on two-dimensional gels (Figure 7). Using inhibition of the precipitation of ^{125}I-labeled alkaline extract (as described for Figure 5) as a quantitative assay, we determined that 95% of the HSS inhibitory activity was contained in the void peak (M_r >350 000) of a AcA-34 gel filtration column (Figure 6), which also contains detectable 43 000-dalton proteins on $NaDodSO_4$ gels (fraction I). One percent of the inhibitory activity was associated with fraction II, which contains substantial quantities

of CPK and actin (based on two-dimensional electrophoresis). Immunoblotting analysis of two-dimensional gels (Figure 7) revealed that fraction II in fact contains trace amounts of protein spots that bind antiserum 1 and migrate in a fashion identical with that observed in the alkaline extract. Thus, the immunoblotting technique is fully capable of visualizing antigens at levels so low that they are undetectable by Coomassie staining. In the case of the alkali extracts of affinity-purified membranes, loading of a total of 0.15 μg of protein yields overexposed spots after 3 h of autoradiography (Figure 7A'). In the case of fraction II, immunoblotting analysis indicates trace amounts of ν_1 spots readily detectable in samples containing 150-fold higher protein loading than the alkaline extract (Figure 7B'). Thus, the relative content of ν_1 in the various fractions, as determined by antibody inhibition (Figure 5), agreed with estimates obtained by the immunoblotting technique.

Discussion

Like other specialized membrane domains, the neuromuscular junction and the homologous synapse in the *Torpedo* electroplax appear to be under the control of cytoskeletal structures. Evidence for this hypothesis is derived from ultrastructural analysis of the cytoplasm subjacent to the postsynaptic membrane by using the rapid freezing technique (Heuser & Salpeter, 1979) as well as conventional transmission electron microscopy after staining with tannic acid, which acts as a mordant for the electron-dense stain uranyl acetate (Cartaud et al., 1981; Sealock, 1980, 1982; Sobel et al., 1980). The structure specifically stained by tannic acid is readily removed by mild alkali treatment (Sealock, 1982; Cartaud et al., 1981), and the treatment concomitantly removes the M_r 43 000 band from membranes (Neubig et al., 1979; Moore et al., 1979). The molecular composition of this band has been the subject of several investigations. It is clear that the M_r 43 000 band is copurified in *Torpedo* electroplax membrane fractions with the nAChR, which is generally accepted as consisting of four integral membrane subunits (which are not solubilized by the alkali treatment and have M_r's of 40 000, 50 000, 60 000, and 65 000). Yet, release of the M_r 43 000 band does not abolish any of the known nAChR activities, such as binding of cholinergic antagonists and agonists or induction of high-affinity agonist binding sites by incubation with agonists over minutes, or the agonist-induced ion-translocation phenomena (Neubig et al., 1979; Moore et al., 1979). Because of its similar electrophoretic mobility to actin, the M_r 43 000 band was initially thought to contain actin as a major constituent (Karlin et al., 1979). This early observation is consistent with the recent localization of cytoplasmic actin in postsynaptic structures at the neuromuscular junction of rat diaphragm (Hall et al., 1981), as well as the reports of copurification of skeletal muscle actin and the nAChR pentameric core (Gotti et al., 1982). The association of actin with receptor-enriched membrane domains during the early embryonic stages raises the possibility that actin may play a role in the clustering or stabilization of nAChR receptors at the neuromuscular junction possibly in association with ν_1 or other specialized components observed in nAChR-enriched membranes.

The sequence of actin has been highly conserved during evolution; only 6% of the amino acid residues are different in actins from sources as evolutionary divergent as *Acanthamoeba castellanii* and rabbit skeletal muscle (Korn, 1978). From the amino acid sequence of rabbit muscle actin (Collins & Elzinga, 1975), we can theoretically expect 36 tryptic peptides (Bremer et al., 1981). With 16 tyrosine residues present and 3 peptide fragments containing 2 tyrosine residues, we can expect 13 peptides with at least one tyrosine residue. This number is close to the 12 major peptides observed on our maps of radioiodinated tryptic peptides. The two peptide differences that we find between ν_3 and rabbit muscle actin could be due to evolutionary amino acid substitutions. Another possibility is also that the peptide difference is similar to the differences generally found between muscle (α) and the cytoplasmic forms of actin (β and γ). While most of the 24–25 substitutions reported between α-actin and cytoplasmic actins occur in neutral amino acids, having only minor effects on the electrophoretic mobility of peptides, a major difference occurs at the amino-terminal end involving among others the omission of one aspartic acid residue, making the cytoplasmic actins slightly more basic than α-actin (Vandekerckhove & Weber, 1978). At the present time, in the absence of N-terminal analysis or sequence data, we cannot conclude whether *Torpedo* electroplax actin is more closely related to muscle or cytoplasmic actin. Whatever the nature of the observed differences, they are not apparent upon one-dimensional peptide mapping analysis of chicken muscle actin and ν_3 (Porter & Froehner, 1983).

We had earlier reported that ν_2 is one of the most abundant proteins in *Torpedo* cytosol (Gysin et al., 1981). Our estimates from densitometric scans of gels and chromatofocusing experiments of different preparations indicated that ν_2 represented 5–15% of the soluble proteins. The identification of ν_2 as CPK is analogous to the situation in muscle tissue, where CPK has been found in concentrations of 10–20% of the total proteins of the sarcoplasm [references in Watts (1973)]. We have shown here that chromatofocusing is a very efficient method for the purification of CPK from *Torpedo* electroplax. This method may also prove applicable to the purification of CPK from other sources.

While this work was in progress, Barrantes et al. (1983) reported CPK activity in AChR-rich membranes from *Torpedo marmorata* electric tissue and the purification of the enzyme from membrane extracts and cytosol. Using immunological and kinetic criteria, they found no differences between the soluble and membrane-associated forms. This is in accordance with our results that showed identical peptide maps and comigration on two-dimensional gels of soluble and membrane-bound ν_2 (Gysin et al., 1981). Using standard electrophoretic procedures used in clinical isoenzyme analysis, we have identified the *Torpedo* electric organ CPK to be of the muscle-specific (MM) type. This is in contrast to the finding of Barrantes et al. (1983) where the electric tissue enzyme was shown to cross-react with antiserum specific for the brain (BB) type.

CPK and ν_1 overlap considerably in isoelectric focusing parameters, with one of the isoelectric focusing variants, ν_{1c}, possessing a p*I* almost identical with that of ν_{2a}, the basic CPK variant [see Figure 7 and Gysin et al. (1981)]. On the other hand, all of the CPK variants migrate ahead of the ν_1 variants during the second $NaDodSO_4$ dimension; in addition, the ν_{1a} and ν_{1b} variants have isoelectric focusing parameters significantly more alkaline than CPK variants. The similarities in the molecular weights of ν_1, CPK, and actin had suggested to us the hypothesis that perhaps there are interrelationships among the three proteins and that they had diverged during evolution to subserve specialized functions. In contrast to this hypothesis, we were unable to detect any major common antigenic determinants. We readily obtained a monospecific antiserum against ν_1 that binds neither actin nor CPK. Thus, the immunochemical evidence supports the hypothesis that ν_1

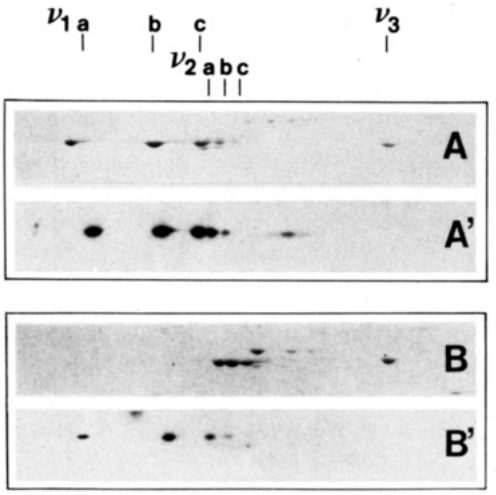

FIGURE 7: Specific reaction of antiserum 1 demonstrated by the immunoblotting technique. Alkaline extract (A, A′) and the cytosol-derived fraction II (B, B′) were analyzed by two-dimensional gel electrophoresis and stained with Coomassie Blue (A, B) or transferred to nitrocellulose membranes and reacted with antiserum 1 (A′, B′). Only the M_r 43 000 regions of the gels are shown; the basic end of the isoelectric focusing dimension is to the left. The p*I* positions of the subunits of ν_1, CPK (ν_2), and *Torpedo* actin (ν_3) are indicated on top. Due to the high sensitivity of the serum, only 0.15 μg of protein was loaded on the gel used for the immunoblot of alkaline extract (A′), compared with 12.5 μg of protein for the Coomassie stain (A). Both gels of the cytosolic fraction II (B, B′) were loaded with 25 μg of protein. The exposure times of the radioautographs after incubation with ^{125}I-labeled protein A were 3 h (for A′) and 12 h (for B′). As is evident from comparison of panels A and A′, the most prominent subunits of ν_1, ν_{1a-c}, and the two less prominent ν_1 subunits immediately to the right of ν_{1c} ($\nu_{1d,e}$) show comparable relative intensity in Coomassie stain and immunoblots with antiserum 1. After longer exposure times of the immunoblots, more acidic p*I* ν_1 variants become apparent, which are not detectable with Coomassie staining (including the spot to the right of ν_{1e} in panel A′). The absence of antiserum reactivity to CPK and actin is demonstrated in panel B′.

is not related to CPK or actin and represents a distinct macromolecule that subserves a unique structural or functional role.

The same conclusion is obtained from an analysis of the amino acid data, which revealed striking differences in the composition of ν_1 and CPK. CPK contains less than half the alanine content and twice the proline and the combined aspartic acid and asparagine contents of ν_1. Other significant amino acid composition differences between the two proteins were observed (Table II). In making comparisons among the amino acid compositions, we neglect here any observed differences in serine, combined glutamine and glutamate, and glycine because of the higher individual blank values observed for these amino acids. The occasional high blank for glycine is likely due to its presence in the electrophoretic system running buffers. In all cases, the blanks have been subtracted to allow specific determinations of the amino acid compositions, which showed good agreement among the determinations. The amino acid compositions of ν_1, CPK, and actin are each distinct from the others: for lysine, the values were respectively 5.9%, 8.0%, and 5.1%; for combined aspartate and asparagine, 7.4%, 14.8%, and 9.1%; for alanine, 10.1%, 4.3%, and 7.6%; for isoleucine, 2.5%, 3.7%, and 8.0% [data from Table II for ν_1 and CPK; rabbit skeletal actin composition from Collins & Elzinga (1975); also compare with similar composition of cytoplasmic actin; Vandekerckhove & Weber, 1978]. The differences in amino acid composition among the three proteins indicate that the proteins are not evolutionarily related.

Both the function and the exact physical linkage of ν_1, CPK, and actin with the nAChR structure are still unexplained. While mostly a soluble enzyme, association of CPK with mitochondria, cytoskeletal elements, and plasma membranes has been widely demonstrated [references in Neumeier (1981) and Erickson-Viitanen et al. (1982)]. In heart muscle, CPK is closely associated with Mg^{2+}-ATPase in myofibrils and with (Na^+, K^+)-ATPase in plasma membrane structures (Saks et al., 1976). It is tempting to hypothesize the existence of an analogous linkage between CPK and ATPase in the *Torpedo* membrane. Recently, Elfman et al. (1982) have shown immunological cross-reactivity between the α-subunit of (Na^+, K^+)-ATPase with a 93 000-dalton band associated with *Torpedo* nAChR membranes. However, no evidence has been obtained up to date showing CPK to be associated with this putative ATPase subunit.

Although there is evidence that ν_1 is closely associated with the nAChR in its membrane environment, no direct evidence for its interaction with the nAChR, e.g., cross-linking via bifunctional reagents, has yet been presented. An antiserum which was prepared against the alkaline extract and that binds to the 43 000-dalton band and preferentially to ν_1 (Porter & Froehner, 1983) specifically stains the cytoplasmic face of *Torpedo* nAChR-enriched membrane domains as well as rat muscle end plates (Froehner et al., 1981). More recently, Cartaud et al. (1982) report that a cytoskeletal structure reminiscent of the erythrocyte cytoskeletal structure is observed in *Torpedo* nAChR-enriched membranes. The cytoskeletal structure is stabilized by the action of the heterobifunctional reagent *p*-azidophenacyl bromide, which reacts with sulfhydryl groups (presumably localized on ν_1) and upon activation with light cross-links ν_1 into higher molcular weight structures. Interestingly, ν_2 and ν_3 (creatine phosphokinase and *Torpedo* actin), observed in the membrane preparations prepared by the method of Sobel et al. (1977), are not cross-linked into the higher molecular weight structures.

It has been observed that removal of ν_1 by treatment with mild alkali renders the nAChR more prone to proteolytic cleavage by trypsin (Klymkowsky et al., 1980). Possibly, ν_1 protects the nAChR from attack by endogenous proteolytic enzymes. Thus, it is possible that binding of ν_1 to the nAChR provides a molecular mechanism for increasing the biological lifetime of the receptor. Should extrajunctional receptors not have ν_1-mediated protection from proteolytic enzymes, this would explain the observed differences in extrajunctional and junctional nAChR turnover rates (Chang & Huang, 1975; Brockes & Hall, 1975). Alternatively, the increase in proteolytic susceptibility could be due to the action of the alkali on the lipids surrounding the nAChR. It has been observed that treatment at pH 11 results in saponification of lipid in the nAChR-enriched membranes to free fatty acids. Thus, it remains a possibility that disruption of membrane structure by the release of fatty acids mediates the observed changes in proteolytic susceptibility of the pentameric core nAChR subunits.

The monospecific antiserum for ν_1, prepared during the course of the present studies, should prove highly useful in further investigations of the biosynthesis and posttranslational modification of ν_1 (Gysin et al., 1982). It should also prove useful in identifying (at the molecular and ultrastructural levels) the properties of the postsynaptic cytoskeletal structure described by Cartaud et al. (1982). The neuromuscular postsynaptic cytoskeleton is likely to prove important in the biological organization of the nAChR in its supramolecular milieu; disruption of this important cytoskeletal structure would be expected to result in dysfunction of the neuromuscular junction, possibly by increasing the nAChR's susceptibility to

stimulation of an autoimmune response, such as occurs during the course of experimental and human myasthenia gravis.

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Registry No. Creatine phosphokinase, 9001-15-4.

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