Acetylcholine Receptor-associated 43K Protein Contains Covalently Bound Myristate

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Abstract. Torpedo electroplaque and vertebrate neuromuscular junctions contain high levels of a nonactin, 43,000-M_r peripheral membrane protein referred to as the 43K protein. 43K protein is associated with the cytoplasmic face of postsynaptic membranes at areas of high acetylcholine receptor density and has been implicated in the establishment and/or maintenance of these receptor clusters. Cloning of cDNAs encoding Torpedo 43K protein revealed that its amino terminus contains a consensus sequence sufficient for the covalent attachment of the rare fatty acid myristate. To examine whether 43K protein is, in fact, myristoylated, mouse muscle BC3H1 cells were metabolically labeled with either [^{35}S]cysteine or [^{3}H]myristate and immunoprecipitated with a monospecific antiserum raised against isolated Torpedo 43K protein. In cells incubated with either precursor, a single labeled species was specifically recovered that comigrated on SDS-PAGE with 43K protein purified from Torpedo electric organ. Approximately 95% of the ^{3}H labeled material released from [^{3}H]myristate-43K protein by acid methanolysis was extractable in organic solvents and eluted from a C_{18} reverse-phase HPLC column exclusively at the position of the methyl myristate internal standard. Thus, 43K protein contains authentic myristic acid rather than an amino or fatty acid metabolite of [^{3}H]myristate. Myristate appears to be added to 43K protein cotranslationally and cannot be released from it by prolonged incubation in SDS, 2-mercaptoethanol, or hydroxylamine (pH 7.0 or 10.0), characteristics consistent with amino terminal myristoylation. Covalently linked myristate may be responsible for the high affinity of purified 43K protein for lipid bilayers despite the absence of a notably hydrophobic amino acid sequence.

The nicotinic acetylcholine receptor (nAchR)[1] is immobilized in stable, high density arrays on the postsynaptic membrane of Torpedo electric organ and vertebrate neuromuscular synapses (2). The molecular mechanisms that are responsible for this distribution are largely unknown but appear to involve both extracellular matrix (2, 34, 36) and intracellular components (21). A distinctive feature of neuromuscular nicotinic cholinergic synapses thought to play a key role in the maintenance of nAchR clusters is a specialized network of structural proteins localized to the cytoplasmic membrane face of the clusters (25, 52). Among proteins that have been identified in this meshwork in muscle are α-actinin (4), filamin (4), vinculin (4), talin (54), and a nonsarcomeric form of actin (24), all known elements of the cytoskeleton of many cell types. In addition, a peripheral membrane protein of 43,000 M_r that appears to be unique to skeletal muscle and electroplaque cells (30) is a prominent component of the synaptic apparatus (39, 45, 59). This latter polypeptide is clearly distinct from actin (45, 60) and is referred to simply as the 43K protein.

1. *Abbreviation used in this paper:* nAchR, nicotinic acetylcholine receptor.

The 43K protein was first described as a major protein of nAchR-rich postsynaptic membranes isolated from Torpedo electric organ (56, 57) where it is found in quantities roughly equal to that of receptor (30). A close association between 43K protein and the nAchR was suggested by the remarkably exact colocalization of these proteins in the electrocyte postsynaptic membrane (53) and the ability of 43K protein to be chemically cross-linked to the β subunit of the nAchR in isolated membrane fragments (9). More recently, evidence for a direct association between 43K protein and the nAchR has been obtained using freeze-fracture immunoelectron microscopy (7). The 43K protein is very tightly bound to the electrocyte membrane, requiring alkaline solutions (pH ⩾11) or the chaotropic agent lithium diiodosalicylate to dissociate it from isolated nAchR-rich membrane fragments (19, 38). Removal of 43K protein by these means does not affect the Ach-activated permeability characteristics of the nAchR (38) but markedly increases the lateral (1, 17) and rotational (49) mobility of the receptor in the plane of the plasma membrane. The skeletal muscle counterpart of Torpedo 43K protein has been localized by immunofluorescence microscopy to the cytoplasmic face of the postsynaptic membrane of vertebrate skeletal muscle synapses (22, 45) as well as to both innervated and aneural nAchR clusters on cultured muscle cells (8, 44). As in Torpedo postsynaptic membranes, there is a precise correspondence in the distribution of immunologically detected 43K protein and nAchR in these cells (44), with both proteins accumulating at newly forming nerve-muscle synapses at the same rate (8). Skeletal muscle 43K protein also resembles the Torpedo protein in being associated with nAchR clusters on receptor-rich membrane fragments and because redistribution of these nAchRs occurs upon its removal with either high pH or lithium diiodosalicylate (5). Thus, the 43K proteins from skeletal muscle and from electrocytes most likely share functional as well as structural similarities.

The amino acid sequence of Torpedo 43K protein was determined by cDNA cloning (20) and direct protein sequencing of purified 43K protein (16). Consistent with the known physical properties of Torpedo 43K protein, the sequence was found to be very rich in cysteine and lacking in classic hydrophobic membrane spanning regions. Interestingly, the amino terminus of Torpedo 43K protein is blocked to Edman degradation (16) and was deduced from the cDNA sequence to be Met-Gly-Gln-Asp-Gln-Thr (20). Recent studies by Frail et al. (19a) demonstrate that the cDNA-derived sequence of mouse muscle 43K protein begins with an identical hexapeptide. Assuming cotranslational removal of the initiator methionine, this sequence contains a combination of features (NH_2-terminal glycine; small, uncharged residues in positions 2 and 5) demonstrated by Towler et al. (63–65) to constitute a consensus sequence for amino terminal addition of the saturated 14-carbon fatty acid, myristate. Myristoylation, unlike palmitylation, is a relatively rare modification that has been described for only a handful of cellularly encoded proteins (65). To examine whether 43K protein contains covalently bound myristate, we have developed a protocol for the metabolic labeling and immunoprecipitation of this protein from the murine muscle cell line BC3H1. BC3H1 cells have been well-characterized in terms of their fatty acylation of proteins (40, 42, 62) and have been shown by LaRochelle and Froehner to contain a 43,000-M_r protein that is specifically recognized by monoclonal antibodies raised against Torpedo 43K protein (31). We report here that 43K protein can be metabolically labeled with [^{3}H]myristate in BC3H1 cells. Chemical characterization of the ^{3}H-labeled moiety of immunoprecipitated 43K protein demonstrated that [^{3}H]myristate is covalently incorporated into the 43K protein as authentic myristate via a hydroxylamine-resistant (presumably amide) bond, as expected for a protein containing NH_2-terminal myristoylglycine. In analogy to its proposed function in certain other proteins, myristate may be involved in the association of 43K protein with the inner surface of the plasma membrane.

Materials and Methods

Anti-43K Serum and Monoclonal Antibodies

Electrophoretically pure 43K protein was isolated from *Torpedo californica* electric organ postsynaptic membranes by preparative SDS-PAGE (16) and used as the immunogen for production of polyclonal anti–43K serum. A New Zealand white rabbit was immunized with complete Freund's adjuvant containing 50 μg purified 43K protein by subcutaneous injection and was boosted at 5-wk intervals, each time with 50 μg 43K protein in incomplete Freund's adjuvant. Characterization of this antiserum is provided in the Results section.

The mouse monoclonal antibody, mAb 19F4a, was generated as described by Bridgman et al. (7) except that partially purified preparations of 43K protein (pH 11 extracts of Torpedo postsynaptic membranes) were used as immunogen. This antibody reacts uniquely with the 43K protein on one- and two-dimensional immunoblots of Torpedo electric organ proteins. Total nAchR α subunit was detected with the rat monoclonal antibody mAb 61 which is specific for the α subunit and has been characterized by Tzartos et al. (66) and Merlie and Lindstrom (37).

Cell Culture and Labeling Conditions

The BC3H1 mouse muscle cell line (50) was grown as described by Merlie and Lindstrom (37). 7-d-old confluent 60-mm cultures of BC3H1 cells were used for all experiments.

BC3H1 cultures were labeled with [^{35}S]cysteine by removing one-half (2.5 ml) of the growth medium and adding 0.25 mCi [^{35}S]cysteine (>600 Ci/mmol; Amersham Corp., Arlington Heights, IL) directly to the remaining medium. Labeling time was 4 h. For labeling with ^{3}H fatty acids, a modification of the procedure of Olson et al. (40) was used. Cultures were rinsed 3 times in DME (high glucose/high bicarbonate formulation) and incubated for 4 h in the same medium supplemented with 5% delipidated and dialyzed FCS, L-glutamine (0.1 mg/ml), 6 mM pyruvate, and either [9, 10-^{3}H(N)] myristate (20–40 Ci/mmol; New England Nuclear, Boston, MA) or [9, 10-^{3}H(N)] palmitate (20–40 Ci/mmol; New England Nuclear). 1/2 mCi of tritiated fatty acid was used per plate.

Preparation of Cell Lysates

At the end of labeling, medium was removed and cultures were rinsed three times with PBS followed by a single wash with "extraction buffer" (0.05 M NaCl, 0.01 M Hepes, 2.5 mM $MgCl_2$, 0.3 M sucrose, 2 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.4) (3). Cultures werre placed on ice and incubated for 2 min at 4°C with 1 ml of extraction buffer with 0.5% Triton X-100 and protease inhibitors (200 μM leupeptin, 0.2 mg/ml α_2-macroglobulin. 50 μg/ml aprotinin, and 500 μM benzamidine). Cells were then scraped from the plate with a rubber policeman and incubated at 4°C for 15 min to solubilize membranes. Preliminary experiments demonstrated that reactivity of the anti-43K serum with 43K protein was markedly enhanced if the Triton-solubilized cell lysates were denatured and alkylated before immunoprecipitation. The lysates were therefore incubated with 0.2% SDS and 10 mM *N*-ethylmaleimide at 4°C for 10 min, after which they were passed three times through a 27-gauge needle to shear DNA released from lysed nuclei. Samples were then diluted with an equal volume of extraction buffer supplemented with 0.5% Triton X-100 and 10 mM *N*-ethylmaleimide before immunoprecipitation.

Immunoprecipitations

For immunoprecipitation with anti-43K serum, samples of cell lysates prepared as described above were first precleared with 100 mg of Immuno-Precipitin (Bethesda Research Laboratories, Gaithersburg, MD; 61) and were then incubated overnight at 4°C in the presence of 0.5% BSA and saturating amounts of antiserum. In general, 5 μl of antiserum (bleed 5) was used to immunoprecipitate 43K protein from one-fifth of the total cell lysate prepared from a confluent 60-mm plate of BC3H1 cells. The resulting immune complexes were precipitated by addition of excess Immuno-Precipitin and after a 20-min incubation at 4°C with mixing, collected by centrifugation. Supernatants were discarded, and the pelleted immunoprecipitates were washed five times by suspension in 1 ml of buffer followed by centrifugation for 5 min in a centrifuge (Eppendorf 5413). The buffer for the first four washes was 0.1 M NaCl, 0.02 M Na borate, 15 mM EGTA, 15 mM EDTA, 0.02% Na azide, 10 mM *N*-ethylmaleimide, pH 8.5, ("immunoprecipitation buffer") supplemented with 0.5% Triton X-100, 0.1% SDS, 0.5% BSA, and 0.5 M sucrose. After the fourth wash pellets were resuspended in immunoprecipitation buffer supplemented with 0.1% SDS and 0.05% Triton X-100 and transferred to a new tube. After centrifugation, supernatants were discarded and the pellets were eluted by boiling for 3 min in SDS-PAGE sample buffer containing 2% SDS and 2% 2-mercaptoethanol. Immuno-Precipitin was removed by centrifugation and the supernatant samples were analyzed by SDS-PAGE. Immunoprecipitations with mAb 61 and mAb 19F4a were conducted identically except that Immuno-Precipitin was preabsorbed with either rabbit anti-rat IgG for mAb 61 or rabbit anti-mouse IgG for mAb F10.

Gel Electrophoresis and Fluorography

Immunoprecipitated or total protein samples were analyzed on SDS-polyacrylamide gels (29) as modified by Carr et al. (16) to resolve the 43K protein from nAchR α subunit, creatine kinase, and actin. Resolving and stacking gels contained 8% acrylamide/0.32% N,*N*-methylene bis acryamide and 4% acrylamide/0.16% N,*N*-methylene bis acrylamide, respectively, and electrode buffer consisted of 0.05 M Tris base, 0.38 M glycine, and 0.15% SDS (16). Gels were processed for fluorography (6) for optimal ^{3}H detection and were exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

HPLC Analysis of ^{3}H-Lipids Covalently Associated with the 43K Protein

Three 60-mm cultures of BC3H1 cells were labeled for 4 h with [^{3}H]myristate and 43K protein was immunoprecipitated from the cell lysate with anti-43K serum. Immunoprecipitated proteins were resolved by SDS-PAGE and the region of the unfixed, undried gel containing 43K protein excised using prestained molecular mass standards (Bethesda Research Laboratories) run in adjacent lanes as a guide. Excised gel slices were rinsed once rapidly with 10% methanol, followed by homogenization in 400 μl of digestion buffer (20 mM glycine, 0.1% Triton X-100, pH 11.0, with one drop of toluene added to retard bacterial growth), and incubated at 37°C in the presence of 400 μg of alkaline protease (type XXI; Sigma Chemical Co., St. Louis, MO) with end-over-end mixing. After 4 h, another 400 μg of alkaline protease was added and the incubation was continued for an additional 10 h. Gel fragments were removed by centrifugation for 15 min in a microfuge and the supernatant, containing digested 43K protein, was supplemented with 20 μl of a 20 mg/ml stock of methyl myristate (Sigma Chemical Co.) in methanol. Samples were then lyophilized, redissolved in 1 ml of 83% methanol, 2 N HCl, and heated in a sealed Reactivial (Pierce Chemical Co., Rockford, IL) for 20 h at 95°C under nitrogen. The resulting hydrolysates were extracted four times with 1 ml of analytical grade petroleum ether and radioactivity in the aqueous and organic phases was determined by liquid scintillation counting. Lipids contained in the combined petroleum ether extracts were separated and identified by reverse phase HPLC as described by Olson et al. (40). Briefly, samples were evaporated to dryness under a steam of nitrogen and resuspended in 250 μl HPLC grade methanol containing 400 μg of methyl palmitate (Sigma Chemical Co.). This was loaded onto a 4.6-mm × 15-cm column (Ultrasphere-ODS C_{18}; Beckman Instruments, Inc., Palo Alto, CA) equilibrated in 80% acetonitrile (American Burdick and Jackson, Muskegon, MI) and eluted isocratically at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals and counted in 6 ml of 3a70B scintillation mixture (Research Products International Corp., Mt. Prospect, IL). The elution positions of the unlabeled methyl myristate and methyl palmitate internal standards were determined by UV absorption at 214 nm.

Hydroxylamine Treatment of Fatty Acylated Proteins

To examine the ability of hydroxylamine to release myristate from the 43K protein, [^{3}H]myristate-labeled 43K protein was immunoprecipitated from metabolically labeled BC3H1 cells, eluted by boiling in SDS-PAGE sample buffer, and incubated with 7 vol of either 1.1 M hydroxylamine, pH 7.0, or 1.1 M Tris, pH 7.0, for 4 h at room temperature. Protein was then precipitated with 20% TCA, washed 4 times with ice-cold acetone, and redissolved in SDS-PAGE sample buffer before analysis by SDS-PAGE. Total fatty acylated proteins in BC3H1 cells were tested for hydroxylamine sensitivity in the same manner, substituting samples of BC3H1 cell lysates labeled with either [^{3}H]myristate or [^{3}H]palmitate for immunoprecipitated 43K. Alternatively, immunoprecipitated [^{3}H]myristate-43K protein or labeled BC3H1 lysates were treated with hydroxylamine after SDS-PAGE by soaking gels for 16 h in 1.0 M hydroxylamine or, as a control, 1.0 M Tris, pH 7.0 or pH 10.0, as described by Olson et al. (40).

Preparation of 43K Protein-enriched Alkaline Extract from Torpedo Postsynaptic Membranes (pH 11 Extract)

nAchR-rich membranes were isolated from the electric organ of *Torpedo californica* using a modification (43) of the procedure of Sobel et al. (57). The 43K protein and other peripheral membrane proteins were extracted from these membranes by incubation at pH 11 (38). Briefly, membrane suspensions in 38% sucrose were sedimented by centrifugation at 100,000 *g* for 20 min and resuspended in water to a concentration of 3 mg protein/ml. The pH was adjusted to 11.0 with NaOH and the preparation incubated at 4°C for 1 h. Membranes were then pelleted as before, after which the supernatant extract was neutralized with 1 M HCl. Any insoluble material was removed from the extract by centrifugation at 100,000 *g* for 20 min and the supernatant was stored at −70°C in single use aliquots. As analyzed by SDS-PAGE and Coomassie Blue staining, ~80% of the protein in the pH 11 extract consisted of the 43K protein with little or no detectable contamination with nAchR subunits. For competition experiments, the equivalent of 90 μl of pH 11 extract (estimated by protein assay and SDS-PAGE to contain ~150 μg of 43K protein) was used per 60-mm dish of cell lysate or 30 μl in vitro translation reaction. No attempt was made to determine the minimum amount of pH 11 extract required for each competition reaction.

In Vitro Transcription, Translation, and Immunoprecipitation of Torpedo 43K Protein

The Eco RI fragment of the cDNA clone 43.1, comprising the coding region of Torpedo 43K protein (20), was subcloned into the Eco RI site of pGEM-1 (Promega Biotec, Madison, WI). Plasmid was purified, linearized by digestion with Bam HI, and transcribed with T7 RNA polymerase in the presence of 1.0 mM m7G(5')ppp(5')G and nucleoside triphosphates (Pharmacia Fine Chemicals, Piscataway, NJ) using the Promega Biotec protocol. The mRNA was purified and translated in a nuclease-treated, methionine-free rabbit reticulocyte lysate system (Bethesda Research Laboratories, Gaithersburg, MD) using 86 mM added potassium acetate and 50 μCi of [^{35}S]methionine according to the supplier's protocol. Translation reactions were diluted 10-fold into PBS containing 0.5% Triton X-100, 10 mM EDTA, 200 μM leupeptin, 0.2 mg/ml α_2-macroglobulin, 50 μg/ml aprotinin, and 500 μM benzamidine and immunoprecipitated with anti-43K serum after a preclearing step as described for BC3H1 cell lysates.

Results

Characterization of Anti-43K Serum

The polyclonal anti-43K serum used throughout this study was raised in a single rabbit that had been immunized with 43K protein isolated from Torpedo electrocyte membranes. Reactivity of this antiserum with Torpedo electric organ proteins was assessed using procedures established for the characterization of monoclonal anti-Torpedo 43K antibodies (7). When Torpedo electric organ nAchR-rich membrane proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the anti-43K serum, a 43,000-M_r single band was detected (data not shown). Two-dimensional electrophoresis of a sample containing a mixture of nAchR-rich membranes and Torpedo cytosol resolved the proteins migrating in the 43,000-M_r region of the gel into several species (Fig. 1 *A*). Among these, only a series of three isoelectric variants of pI 7–8 that are characteristic of Torpedo 43K protein (23, 44, 45) were recognized (Fig. 1 *B*). There was no reactivity with creatine kinase or actin, both of which migrate in one-dimensional gels at a position similar to the 43K protein and are potential contaminants of 43K protein preparations.

Reactivity of the antiserum with 43K protein was further confirmed by its ability to recognize 43K protein synthesized in a cell-free system (Fig. 2). A cDNA encoding Torpedo 43K was transcribed in vitro and translated in a reticulocyte lysate devoid of endogenous translatable mRNA. Under these conditions 43K protein is the only labeled species synthesized. The anti-43K serum immunoprecipitated a labeled protein of ~43,000 M_r from these lysates (Fig. 2, lane *1*) whereas normal rabbit serum (lane *3*) or preimmune serum did not. Immunoprecipitation by the anti-43K serum was blocked by an alkaline extract of Torpedo postsynaptic membranes (pH 11 extract) consisting of 80% pure 43K (lane *2*),

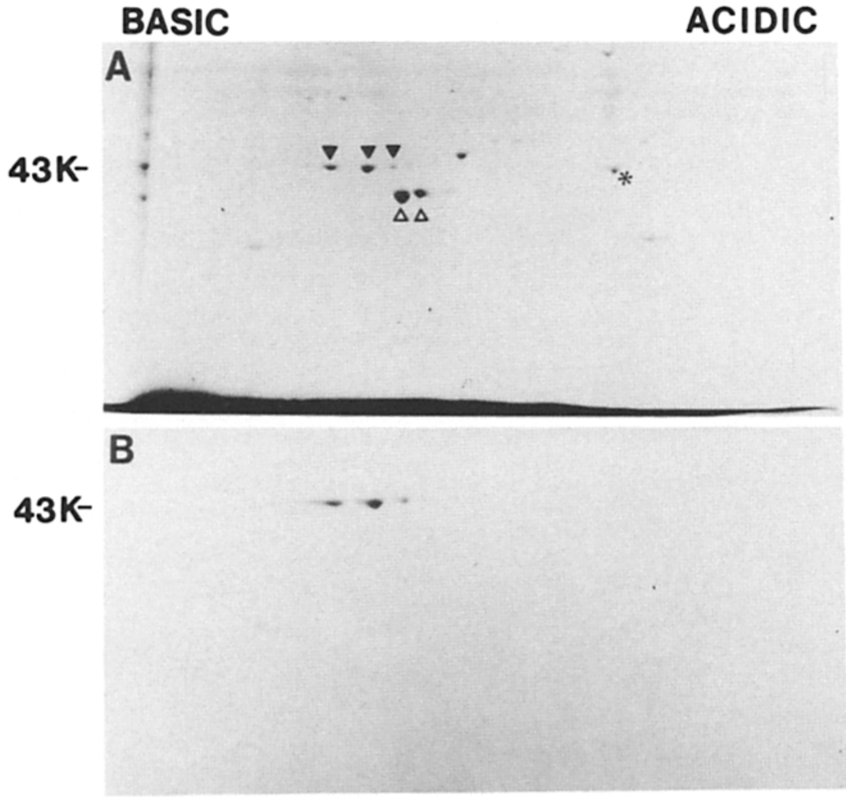

Figure 1. Specificity of anti–43K serum as assayed by two-dimensional immunoblot. A sample containing Torpedo electric organ cytosol combined with nAchR-rich membranes was resolved by two-dimensional gel electrophoresis, and an immunoblot was prepared after electrophoretic transfer of the proteins to nitrocellulose. (*A*) Coomassie Blue stain of the portion of the gel containing immunoreactivity. *Solid triangles*, 43K protein; *open triangles*, creatine kinase; and *asterisk*, actin. (*B*) Blot with antiserum at 1:1,000 dilution.

indicating that the antiserum recognizes authentic 43K protein in a specific manner.

Immunoprecipitation of 43K Protein from Metabolically Radiolabeled BC3H1 Cells

To determine whether 43K protein contains covalently bound lipid, differentiated BCH1 cells were metabolically labeled with [^{35}S]cysteine, [^{3}H]myristate, or [^{3}H]palmitate and detergent lysates of these cells were immunoprecipitated with the polyclonal anti–43K serum (Fig. 3). When such immunoprecipitates were prepared from [^{35}S]cysteine-labeled BC3H1 cells and analyzed by SDS-PAGE and fluorography, a single band that comigrates with 43K protein extracted from Torpedo electric organ nAchR-rich membranes was obtained (Fig. 3, lane *1*). That this 43,000-M_r species is specifically immunoprecipitated authentic 43K protein is supported by the following: (*a*) it is not immunoprecipitated when normal rabbit serum (Fig. 3, lane *2*) or preimmune serum (not shown) is substituted for the anti–43K serum; (*b* it is present in other muscle-derived cell lines and primary cultures of embryonic rat myotubes but not rat H-4-II-E hepatoma cells (data not shown); (*c*) its electrophoretic mobility is distinct from that of the major actin band that is prominent in total BC3H1 lysates (lane *5*); and (*d*) its immunoprecipitation is competitively inhibited by 43K protein–rich Torpedo pH 11 extract whereas the immunoprecipitation of the α subunit of the nAchR by an α-specific monoclonal antibody (mAb 61) is not (Fig. 4). LaRochelle and Froehner have determined a comparable molecular mass for the 43K protein in BC3H1 cells using immunoaffinity chromatography and immunoblotting with anti-Torpedo 43K protein monoclonal antibodies (31).

Identical immunoprecipitations were performed on lysates of BC3H1 cells that were metabolically labeled with [^{3}H]-myristate under conditions reported to result in minimal (∼30%) conversion of exogenously added fatty acids to amino acids in these cells (40). A single species that comigrates with [^{35}S]cysteine-labeled 43K protein was obtained (Fig. 3, lane *3*). Immunoprecipitation of this ^{3}H-labeled band was specific in that it could be competed by pH 11 extracted of Torpedo postsynaptic membranes (Fig. 4, lanes *3* and *4*) and required anti–43K serum (Fig. 3, lane *4*). Moreover, none of the major tritiated proteins of [^{3}H]myristate-

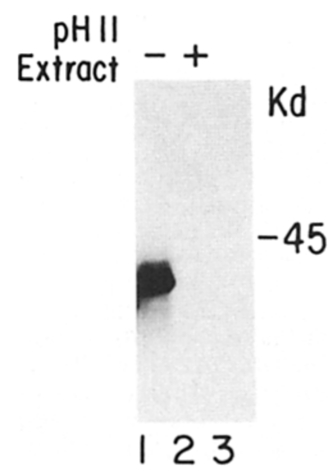

Figure 2. Immunoprecipitation of in vitro–translated Torpedo 43K protein with anti–43K serum. mRNA encoding Torpedo 43K protein was synthesized in vitro from a cDNA template and translated in a nuclease-treated rabbit reticulocyte lysate system supplemented with [^{35}S]methionine. The translation reaction was immunoprecipitated with either anti–43K serum (lanes *1* and *2*) or normal rabbit serum (lane *3*) and analyzed by SDS-PAGE. Anti–43K serum immunoprecipitations were performed in the absence (lane *1*) or presence (lane *2*) of 43K protein partially purified from nAchR-rich Torpedo postsynaptic membranes by pH 11.0 extraction.

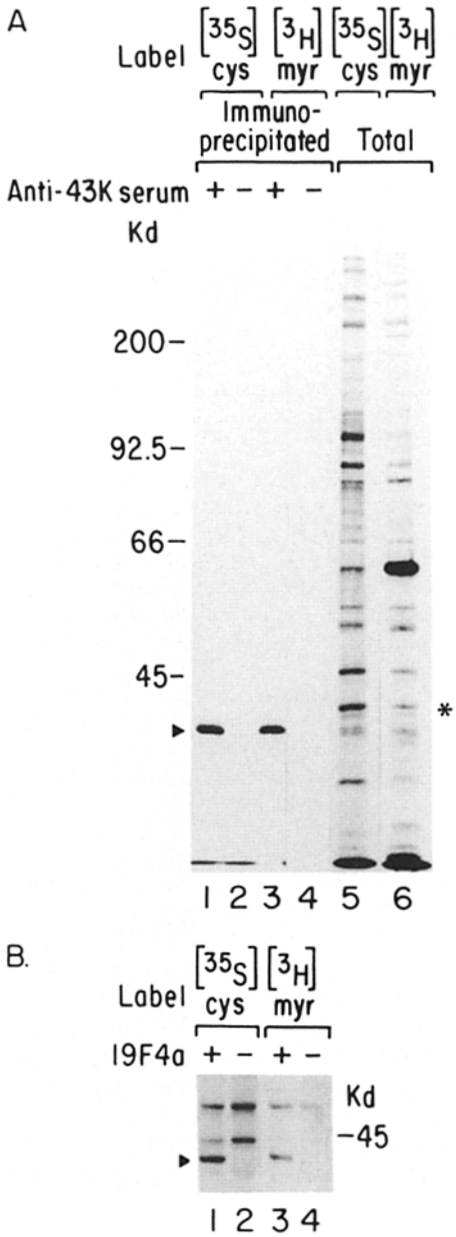

Figure 3. Immunoprecipitation of 43K protein from metabolically labeled BC3H1 cells with anti-43K polyclonal and monoclonal antibodies. BC3H1 cells were labeled for 4 h with either [^{35}S]cysteine or [^{3}H]myristate and lysed as described in Materials and Methods before immunoprecipitation or total protein analysis. Four times as much cell lysate was used per immunoprecipitation from [^{3}H]myristate-labeled cells as from [^{35}S]cysteine-labeled cultures to compensate for the difference in labeling intensity with the two isotopes. (*A*) Immunoprecipitation of [^{35}S]cysteine-labeled (lanes *1* and 2) or [^{3}H]myristate-labeled (lanes *3* and *4*) BC3H1 cell lysates with either polyclonal anti-43K serum (lanes *1* and *3*) or normal rabbit serum (lanes *2* and *4*); total cellular proteins labeled with either [^{35}S]cysteine (lane *5*) or [^{3}H]myristate (lane *6*). The *asterisk* marks the position of the major actin band in lane *5*. (*B*) Immunoprecipitation of [^{35}S]cysteine-labeled (lanes *1* and 2) or [^{3}H]myristate-labeled (lanes *3* and *4*) BC3H1 lysates with either anti-43K mAb 19F4a (lanes *1* and *3*) or an irrelevant (anti-mouse leutinizing hormone) monoclonal antibody prepared similarly (lanes *2* and *4*).

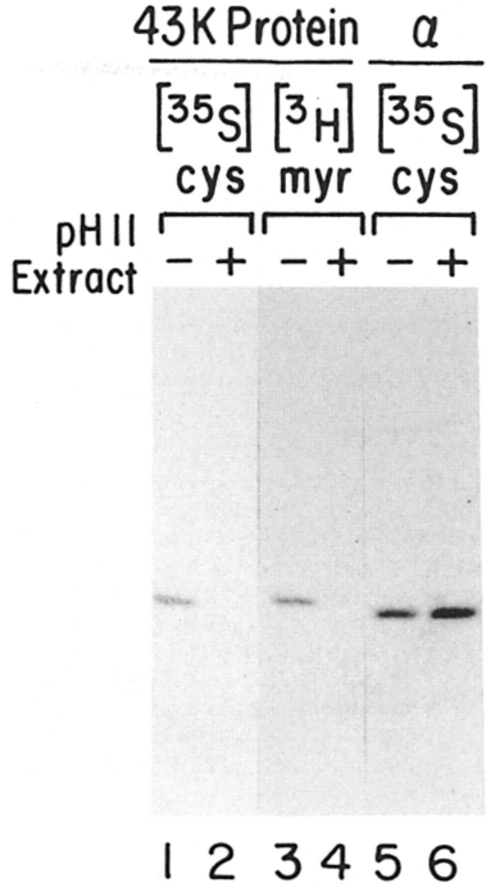

Figure 4. Specificity of immunoprecipitation of [^{35}S]cysteine- or [^{3}H]myristate-labeled 43K protein. BC3H1 cells were metabolically labeled with either [^{35}S]cysteine (lanes *1*, *2*, *5*, and *6*) or [^{3}H]myristate (lanes *3* and *4*), lysed, and aliquots of cell lysates immunoprecipitated with either anti-43K serum (lanes *1–4*) or a monoclonal antibody specific for the α subunit of the nAchR (mAb 61; lanes *5* and *6*). Immunoprecipitations were performed in the absence (lanes *1*, *3*, and *5*) or presence (lanes *2*, *4*, and *6*) of 43K protein partially purified from nAchR-rich Torpedo postsynaptic membranes by pH 11.0 extraction.

labeled BC3H1 total cell lysates (Fig. 3, lane *6*) comigrated with immunoprecipitated 43K protein, making fortuitous nonspecific precipitation of the 43,000-M_r band extremely unlikely. A monoclonal antibody raised versus purified Torpedo 43K protein, 19F4a (Carr, C., G. D. Fischback, and J. B. Cohen, manuscript submitted for publication), also specifically recognized both [^{35}S]cysteine- and [^{3}H]myristate-labeled 43K protein from BC3H1 cell lysates (Fig. 3 *B*). Myristate appears to be incorporated into the 43K protein covalently in as much as the [^{3}H]myristate label remained associated with immunoprecipitated 43K protein after boiling in SDS/2-mercaptoethanol and electrophoresis.

In contrast to the results obtained with [^{3}H]myristate, very little radioactivity became associated with the 43K protein when BC3H1 cells were incubated with [^{3}H]palmitate under conditions that resulted in intense labeling of several other proteins (see Fig. 6 *B*, lane *3* for example). As quantitated by densitometry, the 43K protein was labeled ∼25 times less efficiently with [^{3}H]palmitate than with [^{3}H]myristate (data not shown). Low-level labeling with [^{3}H]palmitate has been reported for several myristoylated proteins (11,

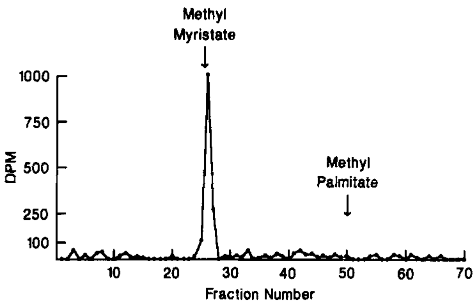

Figure 5. HPLC of fatty acids released from [^{3}H]myristate-labeled 43K protein. 43K protein was immunoprecipitated from three 60-mm dishes of BC3H1 cells that had been metabolically labeled with [^{3}H]myristate for 4 h. The immunoprecipitated 43K protein was isolated by SDS-PAGE, digested exhaustively with alkaline protease, and subjected to acid methanolysis as described in the text. The resulting hydrolysate was extracted with petroleum ether and fatty acid methyl esters contained in the organic phase analyzed by reverse phase HPLC on a C_{18} column. The distribution of radioactivity is compared to the elution position of fatty acid methyl ester internal standards (*arrows*) as determined by UV absorption. Background radioactivity (20 dpm) was subtracted from each fraction.

14, 18, 51) and has been demonstrated in at least two cases to arise from cellular metabolism of a small fraction of [^{3}H]palmitate to [^{3}H]myristate during the labeling period (11, 18). It is therefore likely that [^{3}H]palmitate is incorporated into the 43K protein only after conversion to [^{3}H]myristate or amino acids.

Chemical Identification of the Fatty Acid Covalently Bound to the 43K Protein as Myristate

Although some similarities are apparent, SDS-PAGE analysis of proteins labeled during a 4-h incubation of BC3H1 cells with either [^{35}S]cysteine or [^{3}H]myristate demonstrates that each precursor labels a distinct set of proteins (Fig. 3, lanes *5* and *6*). The difference in these labeling patterns suggests that there is little conversion of [^{3}H]myristate to amino acid metabolites, making it likely that [^{3}H]myristate is incorporated into 43K protein without modification. This was confirmed by chemical analysis of [^{3}H]myristate-labeled 43K protein. Briefly, BC3H1 cells were incubated with [^{3}H]myristate for 4 h and labeled 43K protein was isolated by immunoprecipitation and SDS-PAGE. Gel slices containing [^{3}H]-43K protein were exhaustively digested with alkaline protease, after which the eluted peptides were subjected to acid methanolysis at 95°C for 20 h. The resulting hydrolysate (containing free amino acids and fatty acid methyl esters) was extracted with petroleum ether and the organic phase analyzed by reverse-phase HPLC. All of the ^{3}H radioactivity recovered from the HPLC column (yield = 70–80% of injected radioactivity) migrated at the position of the methyl myristate internal standard and was well-resolved from methyl palmitate (Fig. 5). Moreover, only ~5% of the 43K protein–associated radioactivity partitioned into the aqueous phase after acid methanolysis, indicating negligible conversion of label to amino acids or other water-soluble species before incorporation into 43K protein. Thus, virtually all of the radioactivity recovered from the 43K protein in [^{3}H]myristate-labeled cells is in the form of authentic myristate.

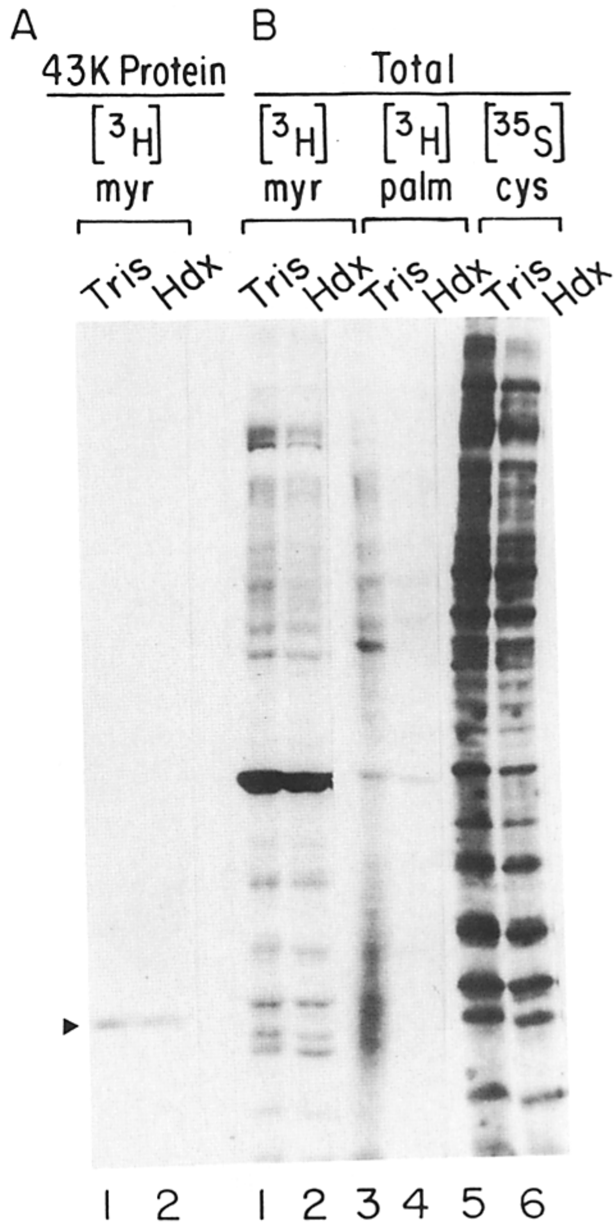

Figure 6. Hydroxylamine stability of the linkage of myristate to 43K protein. BC3H1 cells were metabolically labeled with either [^{3}H]myristate, [^{3}H]palmitate, or [^{35}]cysteine for 4 h and lysed. Equal aliquots of 43K protein immunoprecipitated from these lysates or of total cell lysate were then treated for 4 h at room temperature with either 1 M Tris, pH 7.0, (lanes *1*, *3*, and *5*) or 1 M hydroxylamine pH 7.0 (lanes *2*, *4*, and *6*) before analysis by SDS-PAGE. (*A*) 43K protein immunoprecipitated from [^{3}H]myristate-labeled cells with anti–43K serum. (*B*) Total cellular protein from cells labeled with either [^{3}H]myristate (lanes *1* and *2*), [^{3}H]palmitate (lanes *3* and *4*), or [^{35}S]cysteine (lanes *5* and *6*).

Mode of Attachment of Myristate to the 43K Protein

A fatty acid molecule can be linked to protein via an ester, thioester, or amide bond (41). Palmitate is incorporated into proteins posttranslationally, usually via an ester or thioester linkage (41, 65). In contrast, myristoylation is a cotranslational event (68) in which myristate is typically added to amino terminal glycine residues by means of an amide bond.

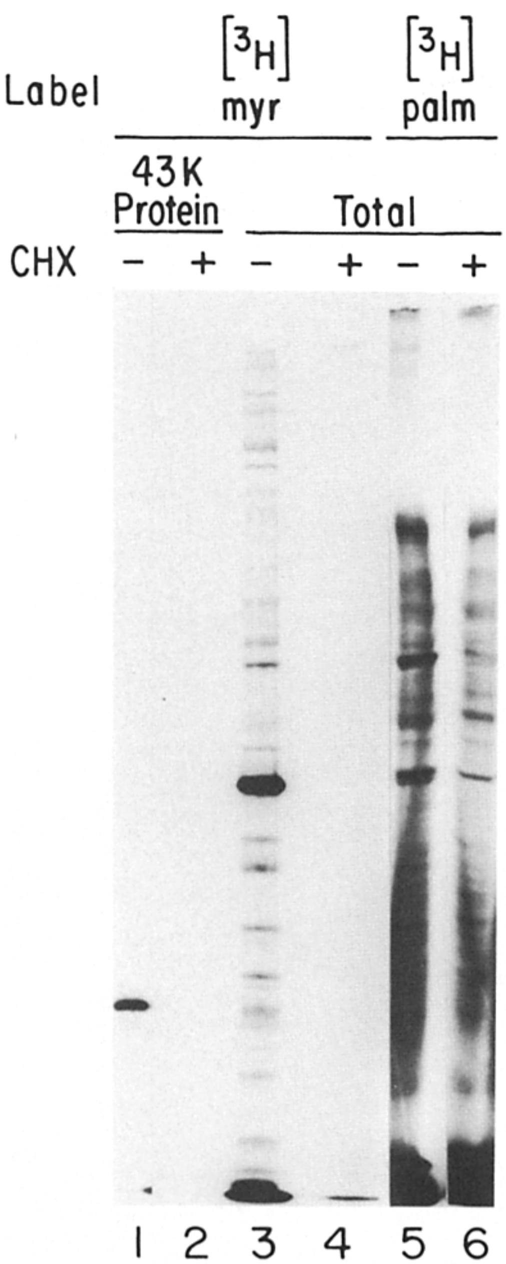

Figure 7. Effect of cycloheximide on the incorporation of [^{3}H]myristate into 43K protein. BC3H1 cells were labeled for 4 h with [^{3}H]myristate (lanes *1–4*) or [^{3}H]palmitate (lanes *5* and *6*) in either the absence or presence of 50 μg/ml cycloheximide. At the end of the labeling period the cells were lysed and either immunoprecipitated with anti–43K serum (lanes *1* and *2*) or analyzed for total labeled protein content (lanes *3–6*). Lanes *1*, *3*, and *5*, control cultures labeled in the absence of cycloheximide. Lanes *2*, *4*, and *6*, cycloheximide added to cultures 10 min before addition of ^{3}H label.

The nature of the linkage of myristate to 43K protein was investigated by examining the stability of this bond to hydroxylamine. Hydroxylamine is a nucleophile that hydrolyzes thioester and (less easily) ester bonds but has little effect on amide linkages (32, 33). A 4-h treatment with 1 M hydroxylamine at pH 7.4 resulted in minimal release of radioactivity from 43K protein immunoprecipitated from [^{3}H]myristate-labeled BC3H1 cells. This was true whether the 43K protein was incubated with hydroxylamine before (in solution; Fig. 6 *A*, lanes *1* and *2*) or after (in a fixed slab gel) SDS-PAGE; similar results were obtained when gel slices containing [^{3}H]myristate-43K protein were soaked for up to 16 h in pH 10 hydroxylamine (1.0 M) or 0.1 M KOH, 40% methanol (data not shown). As expected, the major proteins labeled with [^{3}H]myristate in BC3H1 cells were also resistant to hydroxylamine (Fig. 6 *B*, lanes *1* and 2) whereas identical treatment of lysates of [^{3}H]palmitate labeled cells removed large amounts of radioactivity from most of the prominent ^{3}H-containing proteins (Fig. 6 *B*, lanes *3* and *4*). Based on the criteria of hydroxylamine stability, we conclude that myristate is most likely bound to the 43K protein by an amide-type linkage.

To help distinguish between co- and posttranslational addition of myristate, we examined the effect of inhibition of protein synthesis on the incorporation of [^{3}H]myristate into the 43K protein (Fig. 7). Treatment of BC3H1 cells with 50 μg/ml cycloheximide has previously been shown to reduce protein synthesis in these cells to <5% of control within 2 min (42). This concentration of cycloheximide abolished all detectable incorporation of [^{3}H]myristate into BC3H1 cellular proteins (lane *4*), including immunoprecipitated 43K protein (lane *2*), during a 4-h labeling period. In contrast, considerable labeling of proteins with [^{3}H]palmitate continued in the presence of cycloheximide (compare lane *5* with *6*), indicating addition of [^{3}H]palmitate to preexisting proteins. These results are consistent with cotranslational addition of myristate to 43K protein and suggest that myristate does not turn over during the lifetime of the protein. Similar findings have been reported for several other myristoylated proteins (13, 32, 42).

Discussion

The results of the HPLC analysis presented here clearly demonstrate that the 43K protein is myristoylated in BC3H1 mouse muscle cells. Fatty acid acylation of 43K protein is very specific for myristate, with little or no incorporation of palmitate, and appears to be cotranslational in as much as it is completely inhibited by cycloheximide. In these respects myristoylation of the 43K protein resembles that of several other proteins (11, 13, 32, 35, 42). In virtually all cases examined, protein myristoylation takes place on amino terminal glycine residues via an amide bond (55, 62, 65). Myristate being linked to 43K protein in a similar manner is supported by the following: the presence of a good consensus sequence for myristoylation at the amino terminus of both Torpedo (20) and mouse (19a) 43K protein; the finding that Torpedo 43K protein is blocked to NH_2-terminal Edman degradation (16); and the stability of the association of myristate with 43K protein to hydroxylamine that is indicative of an amide bond. Further experiments will be required, however, before this can be definitively established.

The functional role of myristate is unknown for most proteins. However, point mutations that prevent myristoylation by changing amino terminal glycine residues to alanine, valine, or glutamic acid have important biological consequences in the few cases examined. For example, abolishment of myristoylation of the virally encoded tyrosine protein kinase pp60$^{v\text{-}src}$ makes the protein incapable of stably associating with membranes and renders it nontransforming, presumably by preventing its association with putative membrane-bound substrates whose phosphorylation is necessary for transformation (12, 26, 27). Similarly, mutation of the NH_2-terminal glycine of the gag polyprotein precursor of Mason-Pfizer monkey virus (48) or of Moloney murine leukemia virus (47) appears to completely inhibit virus assembly (47) and/or budding (48) by preventing the association of the gag-encoded structural proteins with the inner plasma membrane. These examples point to a functional role for myristoylation in the anchoring of cytoplasmically synthesized proteins to cellular membranes, presumably via interaction of the myristate moiety with the lipid bilayer. The finding that some myristoylated proteins are soluble rather than membrane bound (15, 42) suggests, however, that acylation with myristate may also serve other purposes such as facilitating specific protein–protein interactions, influencing protein folding, or permitting transient, reversible association of proteins with cellular membranes.

Defining the role of 43K protein myristoylation is complicated by the fact that the function of the protein itself is unknown. The colocalization of the 43K protein with nAchR clusters and the effect of removal of 43K protein on their stability have been interpreted as suggesting that 43K protein is involved in anchoring nAchRs at the postsynaptic membrane, perhaps by acting as a mediator between the receptor and the underlying cytoskeletal network (44, 67). Myristoylation of the 43K protein could aid in this proposed function by allowing direct association of 43K protein with the plasma membrane via lipid–lipid interactions. This possibility is supported by the finding that 43K protein isolated from Torpedo postsynaptic membranes binds tightly and rapidly to pure liposomes (46), even though 43K protein would not be predicted to be particularly hydrophobic on the basis of its amino acid sequence. The apparent lipophilicity of the 43K protein cannot, however, account for why it is detected in situ only at those areas of the plasma membrane that are rich in nAchRs (53). If myristoylation of 43K protein is involved in this preferential subcellular localization, it may be to promote specific interactions between the 43K protein and other proteins such as the nAchR or cytoskeletal components. In this respect it is interesting to note that vinculin (10, 28) and ankyrin (58), both of which have been implicated in plasma membrane–cytoskeleton interactions, contain covalently bound myristate and/or palmitate. The coextensive distribution of the 43K protein with nAchRs observed in vivo most likely results, at least in part, from affinity of the 43K protein for specific proteins as well as for lipids in general; either or both types of interactions might conceivably be mediated by myristate. Dissecting the function of myristate in the 43K protein will be best accomplished by specifically abolishing its myristoylation using site-directed mutagenesis and examining the subcellular distribution of the altered protein. Experiments directed towards this goal (currently in progress) should confirm the molecular site of myristoylation and may also shed light on the role of the 43K protein at the postsynaptic membrane.

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