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Myristic acid is the NH_2-terminal blocking group of the 43-kDa protein of *Torpedo* nicotinic post-synaptic membranes

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The NH_2-terminal blocking group of the 43-kDa peripheral membrane protein (43-kDa protein) of *Torpedo* post-synaptic membranes has been identified as myristic acid. To identify that blocking group pure 43-kDa protein was digested with trypsin and the blocked tryptic peptide was isolated by reverse phase HPLC. That peptide coeluted with and had the same amino acid composition as a synthetic peptide, myristoyl-Gly-Gln-Asp-Gln-Thr-Lys, the structure of the amino terminus predicted from the protein sequence deduced from a cDNA clone. The presence of myristate was confirmed by the precise molecular mass of the peptide, 886.5266, determined by fast atom bombardment mass spectroscopy.

Postsynaptic membrane; Protein, 43 kDa; Myristoylation, N^{α}-; (*Torpedo*)

1. INTRODUCTION

Nicotinic acetylcholine receptors (AChRs) are concentrated at high surface density in the post-synaptic membranes of vertebrate skeletal muscle endplates and *Torpedo* electrocytes (review [1]). The mechanisms responsible for this distribution are unknown but are likely to involve interactions between AChRs and associated peripheral proteins. In postsynaptic membranes isolated from *Torpedo* electric tissue the rotational and translational mobilities of AChRs are severely restricted [2,3] with receptor mobility increased after removal of peripheral proteins by exposure to alkaline pH [4]. The predominant peripheral protein in these membranes is a polypeptide of 43 kDa (43-kDa protein) present on the cytoplasmic aspect of the membrane [5,6] in close proximity to AChRs [7,8]. In the electrocyte the 43-kDa protein is precisely colocalized with AChRs [9] and it is present at equimolar amounts with AChR [10]. An immunologically related determinant is associated with AChRs at vertebrate neuromuscular junctions and in cultured muscle cells [11–14].

The amino acid sequence of *Torpedo* 43-kDa protein, determined by direct protein sequencing [15] and by cDNA cloning [16], indicates that it is a novel protein unrelated to known cytoskeletal proteins. The amino terminus of 43-kDa protein was resistant to Edman degradation, so neither the nature of the blocking group nor the sequence of the amino terminal tryptic peptide was determined chemically. That sequence was predicted from the cDNA sequence to be M-G-Q-D-Q-T-K [16]. If the initiator methionine is removed, that sequence contains the features matching the known specificity of protein *N*-myristoyl transferase [17]. N-terminal myristoylation is a relatively rare modification that can be important for the interaction of otherwise soluble proteins with the plasma membrane [18].

The myristoylation of 43-kDa protein in mouse muscle BC3H1 cells has been described [19] although the location of myristate within the primary structure was not identified. In this letter, we present the isolation of the blocked tryptic pep-

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tide predicted to be the amino terminus of *Torpedo* 43-kDa protein through comparison of its properties on reverse phase HPLC with a synthetic myristoylated N-terminal peptide. The confirmation of myristate as the blocking group on this peptide is achieved by fast atom bombardment mass spectroscopy.

2. MATERIALS AND METHODS

2.1. *Isolation of 43-kDa protein*

Carboxamidomethyl 43-kDa protein ([^{14}C]CAM-43K) was isolated as described [15] from AChR-rich membranes prepared from *Torpedo californica* electric organ. Briefly, peripheral proteins were extracted from membrane suspensions (4 mg protein/ml) by exposure to pH 11 for 1 h at 25°C. Freshly prepared alkaline extract was reduced with dithiothreitol and then reacted with [^{14}C]iodoacetamide. Electrophoresis on polyacrylamide slab gels followed by electroelution was used to isolate [^{14}C]CAM-43K from other peripheral proteins.

2.2. *Peptide synthesis*

The hexapeptide (GQDQTK) proposed to represent the amino terminus of 43-kDa protein [16] as well as N^{α}-myristoyl and N^{α}-palmitoyl derivatives were synthesized by solid-phase methods [20] on an Applied Protein Technologies (Cambridge, MA) PSS-80 peptide synthesizer. All amino acids were coupled in dichloromethane/dimethylformamide as 1-hydroxylbenzotriazole (HOBT) active esters that were generated in situ by mixing equimolar amounts of protected amino acid, 1,3-diisopropylcarbodiimide, and HOBT in the coupling solvent. N^{α}-Myristoylation and N^{α}-palmitoylation were accomplished similarly. Deprotection and cleavage from the resin with HF were performed by Immuno-Dynamics Inc. (San Diego, CA). The peptides were purified by reverse-phase HPLC on a 2.5 × 22 cm Vydac column using a gradient of water-acetonitrile containing 0.09% trifluoroacetic acid (TFA). Purity of the peptides was confirmed by analytical HPLC, amino acid analysis, and microsequencing.

2.3. *Isolation of blocked N-terminal peptide*

Tryptic digestion was performed on 160–200 μg [^{14}C]CAM-34K using the conditions described [15]. The separation of the tryptic peptides was carried out on a Vydac C_4 column at a flow rate of 1 ml/min using a linear water-acetonitrile gradient [15]. Peptides whose elution positions were similar to that of the synthetic myristoylated peptide were repurified on a Vydac C_{18} column using a 25–60% acetonitrile gradient.

2.4. *Fast atom bombardment (FAB) mass spectrometry*

FAB mass spectrometry was conducted using a ZAB-SE double-focusing mass spectrometer equipped with an 11-250J data system (VG Instruments, Danvers, MA). Both synthetic and authentic samples were analyzed by dispersion of an acetonitrile solution in ~2 μl of glycerol on the FAB probe tip. Each mass spectrometric experiment used 100–200 pmol of peptide. Samples were introduced into the mass spectrometer and bombarded with a xenon atom beam with 8 keV energy. Mass spectra were obtained by scanning the mass range 1000–100 at 15 s/decade. Subsequently, a multi-channel acquisition mode was used for molecular mass determination of the authentic material. For these experiments the mass range 800–1000 was scanned linearly at the rate of 10 s/scan. Peak matching experiments were performed using a dual tipped FAB probe. A mixed cesium/rubidium iodide salt preparation was used as a source of reference ions.

3. RESULTS

In a previous study [15] we demonstrated the separation of tryptic peptides of 43-kDa protein by reverse-phase HPLC. To identify the appropriate region of the gradient in which to search for the predicted blocked N-terminal tryptic peptide, the retention properties of synthetic peptides were used as a reference. When placed on a C_4 column and eluted under standard conditions, N^{α}-myristoyl-GQDQTK eluted at 37% acetonitrile while N^{α}-palmitoyl-GQDQTK eluted 8 min later at 41% acetonitrile (fig.1). The peptide with a free amino terminus was not retained on a C_4 column (data not shown). Fig.2 shows the HPLC separation of 4 nmol of [^{14}C]CAM-43K after digestion with trypsin in comparison with the elution of N^{α}-myristoyl-GQDQTK (solid arrow, fraction 138). Material in the two nearest peaks, fractions 137 and 142, were each collected and refractionated on a C_{18} reverse-phase column (fig.3). The material from fraction 137 was resolved into two major peaks, both of which had elution times well resolved from that of the synthetic peptide (fig.3B). In contrast, only a single major peak was recovered from fraction 142, and that material had the same retention time as the synthetic myristoylated peptide on the C_{18} column (fig.3A), and also when rechromatographed on the C_4 column.

The amino acid composition of the peptide identified in fig.3A was determined and compared to that of the synthetic myristoylated peptide which had been treated identically by passage over both HPLC columns. The authentic peptide contained the same amino acids as the synthetic peptide (table 1) and in the same relative ratios: two glutamates for every one aspartate, glycine, threonine and lysine residue. From a tryptic digest of 4.7 nmol [^{14}C]CAM-43K, ~1.7 nmol of peptide was recovered. Acid hydrolysis caused the deamidation of the glutamine residues in the syn-

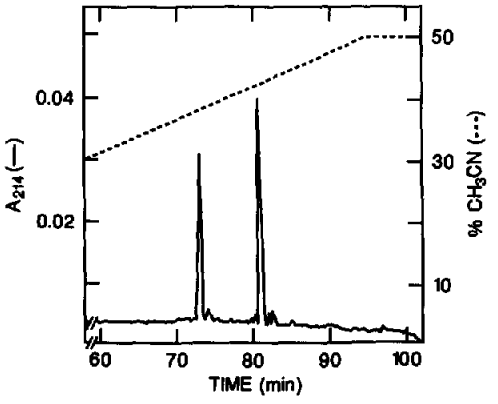

Fig.1. Reverse-phase separation of 1.5 nmol each of synthetic N^{α}-myristoyl-GQDQTK and N^{α}-palmitoyl-GQDQTK on a Vydac C_4 column with 0.1% TFA in water as solvent A and 0.09% TFA in acetonitrile as solvent B. The separation was monitored by measuring the absorbance at 214 nm. After an initial wash with 5 ml of 5% solvent B, peptides were eluted with a linear gradient from 5% to 50% in 90 min. Flow rate was 1.0 ml/min.

thetic peptide and presumably in the isolated 43-kDa peptide as well. Confirmation of glutamine instead of glutamate residues in the tryptic peptide might be obtained from sequence analysis. However, when 100 pmol of peptide were subjected to the Edman degradation, as expected no released amino acids were detected. Thus the amino terminus of this peptide, like that of intact 43-kDa protein [15], was blocked.

To determine unambiguously that myristic acid was the group blocking the amino terminus of the

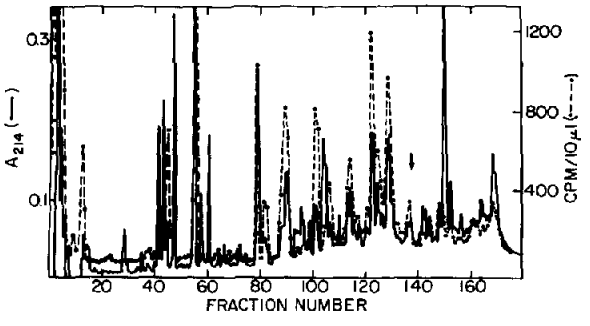

Fig.2. Reverse-phase separation of a digest of [^{14}C]CAM-43K after cleavage with trypsin. [^{14}C]CAM-43K (4 nmol) was digested as described [15], and the digest was resolved on a C_4 column under conditions identical to those in fig.1. Radioactivity of peptides was measured in 10 μl aliquots of each fraction (0.5 ml). The arrow indicates the elution position of synthetic N^{α}-myristoyl-GQDQTK (fig.1).

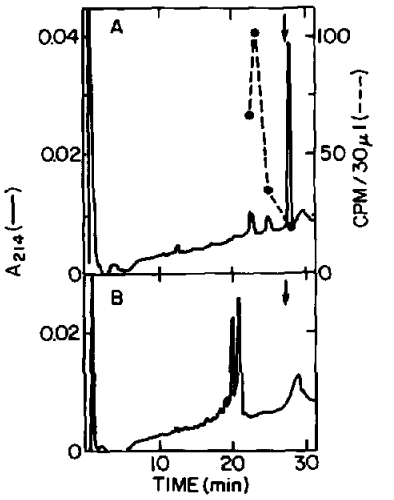

Fig.3. Rechromatography on a Vydac C_{18} column of two fractions (142 in A and 137 in B) from the C_4 separation of a tryptic digest of [^{14}C]CAM-43K (fig.2). The peptides were eluted with a linear gradient from 25% to 60% in 35 min. The flow rate was 1 ml/min and fractions corresponding to *A* peaks were collected manually. The arrow indicates the elution position of synthetic N^{α}-myristoyl-GQDQTK on C_{18} using the same gradient conditions.

tryptic peptide, FAB mass spectrometry was used to determine precisely the mass of the isolated peptide. N^{α}-Myristoyl-GQDQTK has a molecular mass of 885 and the FAB mass spectrum of the synthetic peptide and of the isolated tryptic peptide each had the $[M+H]^+$ protonated molecular signal at *m/z* 886. The molecular region of the isolated tryptic peptide is shown in fig.4. With the quantities of material available (typically 100–200 pmol

Table 1

Amino acid composition of isolated, blocked 43-kDa peptide and of synthetic Myr-GQDQTK after C_4 and C_{18} columns[a]

Amino acid	pmol[b] (ratio)	
	Synthetic	Authentic
Asp	140 (1)	122 (1)
Glu	293 (2.09)	269 (2.20)
Gly	126 (0.90)	106 (0.87)
Thr	147 (1.05)	132 (1.08)
Lys	137 (0.98)	126 (1.03)

[a] Compositional analysis determined as described [15] by derivatization of acid hydrolysates with phenylisothiocyanate

[b] Aliquots of 20 μl of synthetic peptide and 45 μl of isolated tryptic peptide were hydrolyzed; background levels of other amino acids in samples were less than 8 pmol

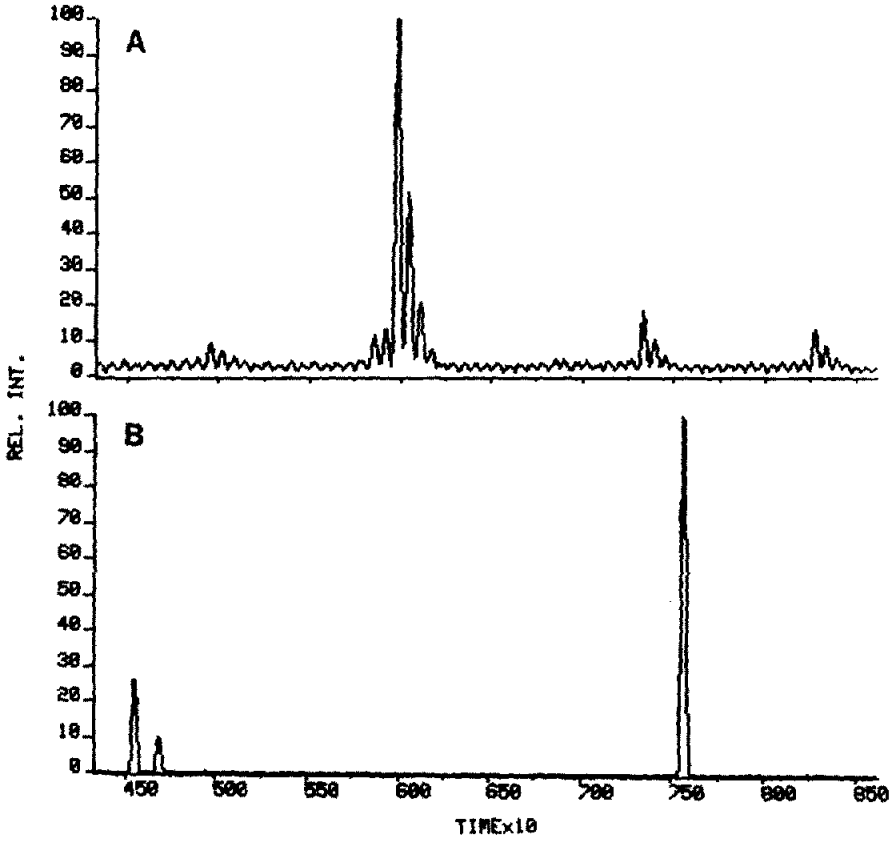

Fig.4. FAB mass spectra of the molecular region of the N-terminal tryptic peptide isolated from *Torpedo* 43-kDa protein (top) and the CsRbI reference (bottom). Spectra are normalized to the largest peak in the spectrum and are plotted against arrival time (in ms following the scan initiation). The most intense peak in the peptide spectrum is at m/z 886 corresponding to the $[M+H]^+$ protonated molecule. The intensities of the ^{13}C isotope signals associated with $[M+H]^+$ are in agreement with calculated intensities. The weaker ions at higher masses correspond to $[M+Na]^+$ at m/z 906 and $[M+K]^+$ at m/z 924.

per analysis) there were no structurally significant fragment ions visible at lower masses. The only other ions in the spectra at lower masses were due to the expected matrix ions from glycerol. To further confirm the composition of the isolated tryptic peptide, the protonated molecular signal was peak matched against the reference ion ($Cs\ ^{85}RbI_3$, mass 864.3415), which was obtained by bombardment of a mixed cesium/rubidium iodide salt. The measured mass, determined as the mean of three experiments, was 886.5266 which compares very well with the calculated mass of the protonated molecule of the N^{α}-myristoyl hexapeptide ($C_{40}H_{72}N_9O_{13}$), 886.5250. Thus the composition of the peptide isolated from the tryptic digest of 43-kDa protein was established as that expected for N^{α}-myristoyl-GQDQTK.

4. DISCUSSION

The results reported here establish that the 43-kDa protein of *Torpedo* post-synaptic membranes joins the growing number of proteins modified by N_{α}-myristoylation [18]. Since all the 43-kDa protein isolated from post-synaptic membranes is resistant to Edman degradation and myristoyl peptide was isolated at good yield (~25%) from the tryptic digest of 43-kDa protein, it is likely that all the 43-kDa protein in the post-synaptic membrane is N^{α}-myristoylated. In *Torpedo* electric organ 43-kDa protein is present at a one to one stoichiometry with AChR [10] and results of immunoelectronmicroscopy establish that its distribution is coextensive with that of AChR [9]. These observations raise the question

whether the N^{α}-myristoylation is important for the interaction of the protein generally with the plasma membrane or specifically with the AChR. Since 43-kDa protein can be released from the postsynaptic membrane by exposure to pH 11 [4], without modification of the NH_2-terminus [15], it is unlikely that the myristate of 43-kDa protein functions simply as a hydrophobic anchor interacting with phospholipid. Capsid protein VP4 of picornaviruses is N^{α}-myristoylated, and in poliovirus where the location of myristate has been determined by X-ray crystallography [21], each myristyl group interacts with amino acid side chains both in VP4, the internal protein, and in the surface protein VP3. Thus the myristate in 43-kDa protein, an internal protein, may be directly involved in interactions with AChR, a surface protein.

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