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Two isoforms of the muscle acetylcholine receptor α-subunit are translated in the human cell line TE671

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We have previously reported the existence of 2 forms of mRNA for the human muscle acetylcholine receptor (AChR) α-subunit, thought to be generated by alternate splicing of a primary transcript and to encode 2 α-subunit protein isoforms [1]. The 2 predicted α-subunit isoforms, differing by the insertion of 25 amino acids at position 58/59, have been synthesized from cRNA transcripts using rabbit reticulocyte lysates: these protein isoforms could be differentiated by immunoprecipitation using antibodies raised against synthetic peptides. The antibodies were used to demonstrate translation of both AChR α-subunit isoforms in the rhabdomyosarcoma (muscle) cell line TE671, in an approximate 1:1 ratio.

AChR: α-Subunit; Isoform; Translation; TE671 cell

1. INTRODUCTION

Muscle nicotinic acetylcholine receptors (AChR) mediate synaptic transmission at the neuromuscular junction. Structurally, each AChR is a pentamer, with subunit stoichiometry α_2, β, γ/ε, δ, creating a central ion channel that crosses the postsynaptic membrane. The human AChR is of particular interest because it is the target for autoimmune attack in the disease myasthenia gravis, the main focus for the antibody binding being the α-subunit.

The genes encoding the AChR form part of the gene superfamily of ligand-gated ion channels that also include receptors for glycine and γ-aminobutyric acid. There is a high level of sequence homology across species for the AChR α-subunit [2], implying strong evolutionary constraints. However, in contrast with other species, 2 forms of mRNA, thought to be generated by alternate splicing, may encode the human AChR α-subunit [1]. We show here that both protein isoforms can be synthesized and glycosylated in a reticulocyte cell lysate system to generate polypeptides of apparent mol. wts. of 44 and 46 kDa when visualized by SDS/PAGE. We also show for the first time that the isoforms are present in the human cell line TE671 which expresses functional muscle type AChRs [3]. In each case the larger isoform binds antibodies specific for the 25 amino acids contributed by the extra exon P3A.

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2. MATERIALS AND METHODS

2.1. *cRNA synthesis and in vitro translation*

cDNA clones encoding the human AChR α-subunit + P3A and α-subunit − P3A were subcloned into the *Eco*RI site of pSP19 (BRL) and the *Hin*cII site of pAM19 (Amersham), respectively. Both forms of α-subunit cRNA were transcribed from linearised plasmid templates with SP6 RNA polymerase (Pharmacia), and translated in vitro using nuclease treated rabbit reticulocyte lysates in the presence of canine microsomal membranes (Promega) and L-[^{35}S]methionine (Amersham) according to the manufacturer's instructions.

2.2. *TE671 cells*

Cells were grown under conventional conditions as previously described [3]. Approximately 4×10^7 cells (per 800 ml flask) were labelled for 8 h with 500 μCi L-[^{35}S]methionine (Amersham). PBS-washed cells were extracted in 1 ml of 1% Triton X-100/20 mM phosphate buffer (pH 7.4)/1 mM PMSF and the supernatant stored at −70°C until use.

2.3. *Preparation of antibodies*

Peptides were synthesized using Fmoc chemistry on an LKB Biolynx 4175 peptide synthesizer, and polyclonal antisera against them raised in rabbits. Peptides were then conjugated to CNBr-activated sepharose 4B (Pharmacia) and specific antibodies against each peptide purified by affinity chromatography.

2.4. *Immunoprecipitations*

The immunoprecipitation protocol was essentially as described by Sumikawa and Miledi [4]. [^{35}S]Methionine-labelled TE671 cell extracts or reticulocyte lysates were heated to 100°C in 1% SDS/5 mM EDTA/PBS for 3 min and then diluted with 4 vols. of buffer A (60 mM Tris-HCl (pH 7.6)/5 mM EDTA/190 mM NaCl/1.25% Triton X-100). Affinity-purified anti-AChR α-subunit peptide antibodies are added and incubated overnight at 4°C. Immunocomplexes were precipitated with formalin-fixed *Staphylococcus aureus* cells (BRL), washed 3-times with NETS buffer (10 mM Tris-HCl (pH 7.4)/1 mM EDTA/100 mM NaCl/0.5% NP-40) and analysed by SDS gel electrophoresis after solubilisation in SDS/PAGE sample buffer [5].

3. RESULTS AND DISCUSSION

Fig. 1 shows the proposed structure of the 2 isoforms, including the 25 amino acids of P3A inserted between

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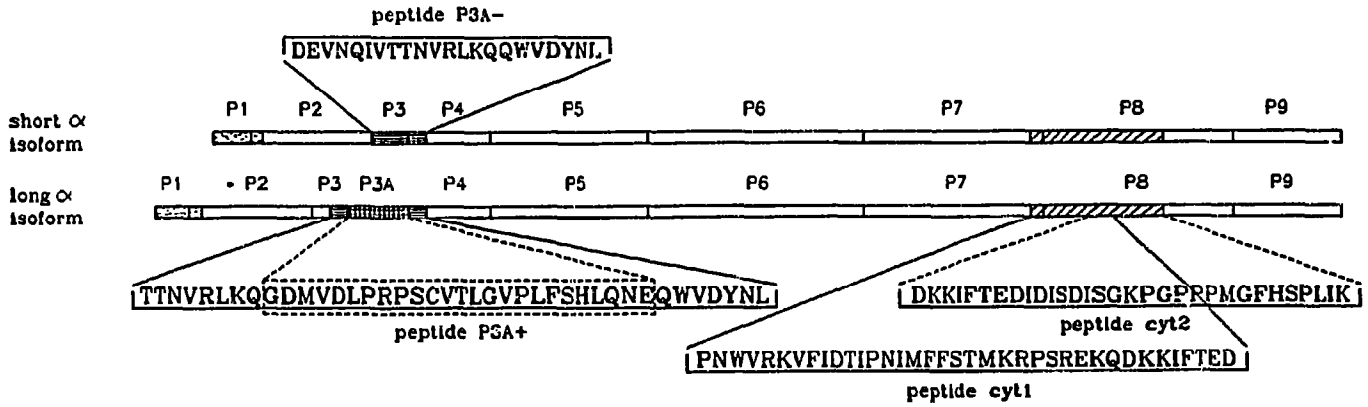

Fig. 1. Location of the amino acid sequences within the AChR α-subunit protein isoforms for the synthetic peptides used to raise antisera. P1–P9 refer to the gene exons [6], with the long isoform containing exon P3A. The lightly shaded area corresponds to the signal peptide, removed during protein maturation.

amino acids 58/59, and the sequence of the peptides used to raise antibodies. Peptide 3A+ has short sequences from exons P3 and P4 as well as P3A, whereas peptide P3A− represents amino acids 45–65 without the P3A sequence. Two overlapping peptides, cyt 1 and cyt 2, (thought to be located cytoplasmically in the native structure) were used to raise antibodies that would bind readily to both forms of the α-subunit.

Dot blot assays (data not shown) were used to confirm that antisera were specific for the immunising peptide(s), and that antisera raised against peptide P3A+ (anti-P3A+) did not bind to peptide P3A−. Antisera to peptides cyt 1 and cyt 2 (anti-cyt 1,2) were also able to precipitate native human AChR labelled with [α-^{125}I]bungarotoxin (α-BuTx) whereas anti-P3A+ could not.

In order to demonstrate isoform-specific antibody-binding to full length α-subunit we generated the isoforms by translating cRNAs using rabbit reticulocyte lysates. Fig. 2 shows immunoprecipitations of the translation products. Two major gel bands were seen for each α-subunit isoform because glycosylation using pancreatic microsomal membranes is not 100% efficient; the upper band is the glycosylated form. Anti-cyt1,2 antibodies bound both isoforms, whereas anti-P3A+ antibodies only bound the larger isoform. The level of the larger α-subunit isoform precipitated was always less with anti-P3A+ than with anti-cyt 1,2.

A similar assay was carried out on [^{35}S]methionine-labelled protein extracts from TE671 cells. Accordingly, Fig. 3a (lane 3) shows that the anti-cyt 1,2 antibodies precipitated bands of M_rs corresponding to the larger and smaller α-subunit isoforms, whereas anti-P3A+ antibodies (lane 4) precipitated only the larger α-subunit form. Other protein bands visible in these tracks appear to have precipitated non-specifically due to their abundance, since they are seen in diluted TE671 extract, and are also brought down by purified control rabbit IgG. Since anti-P3A+ immunoprecipitates only one band corresponding to α-subunit protein, it is unlikely that the 2 bands seen using anti-cyt 1,2 represent a single isoform before and after glycosylation. Thus, both AChR α-subunit isoforms are translated in vivo in

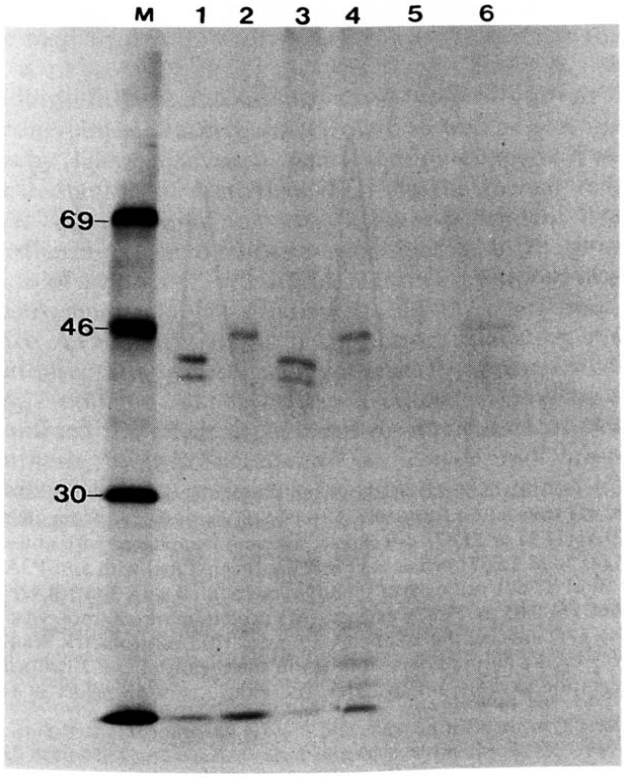

Fig. 2. cRNAs encoding the AChR α-subunit isoforms translated using rabbit reticulocyte lysates with the addition of canine microsomal membranes and [^{35}S]methionine. Autoradiograph of samples after 10% SDS/PAGE: (1) translation of cRNA-P3A, 2.5 μl; (2) translation of cRNA + P3A, 2.5 μl; (3) 5 μl cRNA − P3A translation, immunoprecipitated with anti-cyt 1,2; (4) 5 μl cRNA + P3A translation, immunoprecipitated with anti-cyt 1,2; (5) 5 μl cRNA − P3A translation, immunoprecipitated with anti-P3A+; (6) 5 μl cRNA + P3A translation, immunoprecipitated with anti-P3A+; (M) protein markers (mol. wts. in kDa).

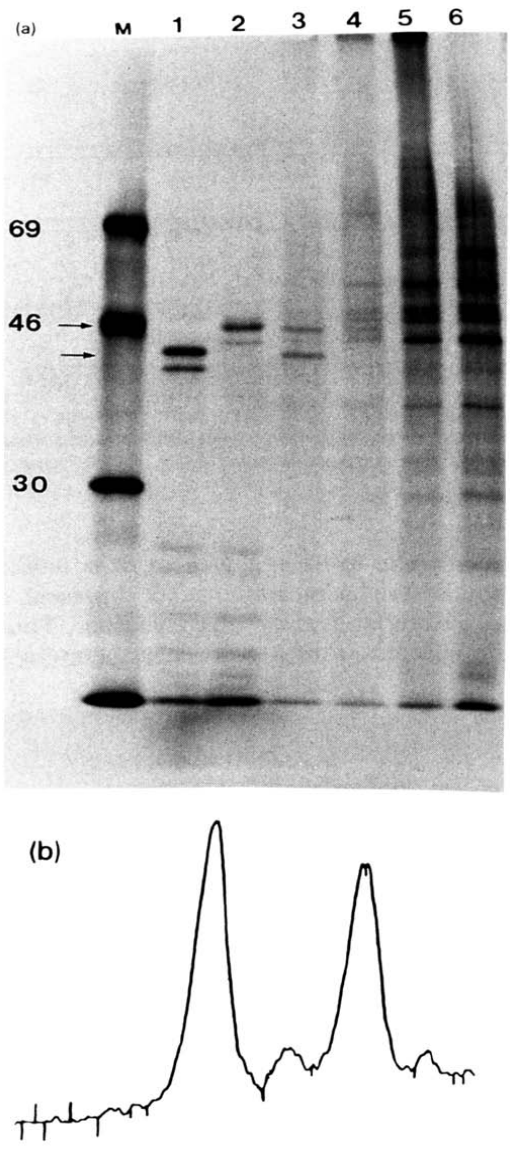

Fig. 3. (a) Immunoprecipitation from [^{35}S]methionine-labelled TE671 cells; (1) translation from cRNA − P3A; (2) translation from cRNA + P3A; (3) 50 μl TE671 cell extract immunoprecipitated with anti-cyt 1,2; (4) 50 μl TE671 cell extract immunoprecipitated with anti-P3A+; (5) 50 μl TE671 cell extract immunoprecipitated with control rabbit serum; (6) 0.05 μl TE671 extract; (M) protein markers (mol. wts. in kDa), (→) indicate the α-subunit isoforms. (b) Densitometric scan of lane 3 of the autoradiograph in (a) in the region of the 2 isoforms. Integration gave the relative area under the 2 isoform peaks as 44.3 and 46.8%, respectively.

TE671 cells; moreover, the isoforms are present at a ratio of approximately 1:1 (Fig. 3b), which matches the relative levels of mRNA previously reported [1]. Since studies by several groups [8–11] have shown that the major control of AChR synthesis is at the transcriptional rather than translational level, and mRNA containing P3A has been detected in muscle, it is likely that both isoforms are translated in vivo in human muscle.

Although the translation of the P3A isoform in TE671 cells has been demonstrated it has not been shown to be assembled and inserted into the membrane, nor has the function of the P3A insert been established. A high level of unassembled α-subunit that has the characteristics of an assembly intermediate with the ability to bind α-BuTx has been shown in TE671 cells [12,13]. Such an intermediate has not been seen in BC3H-1 cells [14] or cultured rat [15] and chicken myotubes [16]. Furthermore, injection of human AChR α-subunit cRNA into *Xenopus* oocytes led to the formation of an α-BuTx-binding intermediate not seen after injection with *Torpedo* α-subunit cRNA [13]. Other examples of alternate splicing within the ligand-gated ion channel gene superfamily [17,18] generate potential phosphorylation sites within their cytoplasmic segments. However, the presence of the P3A sequence in the extracellular domain, within a region of the subunits that is relatively highly conserved throughout this gene superfamily, suggests some other function, perhaps involved with AChR assembly.

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