Alternative splicing generates multiple mRNA forms of the acetylcholine receptor γ-subunit in rat muscle

Alfredo Villarroel*

Department of Physiology and Biophysics, Dalhousie University Faculty of Medicine, 3R1 Tupper Medical Building, Halifax, N.S. B3H 4H7, Canada

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Abstract The fetal type acetylcholine receptor, composed of the αβγδ subunits, has shown a highly variable channel kinetics during postnatal development. We examine the hypothesis whether such a variability could result from multiple channel forms, differing in the N-terminus of the γ-subunit. RT-PCR revealed, in addition to the full-length mRNA, three new forms lacking exon 4. One of them in addition lacks 19 nucleotides from exon 5, predicting a complete subunit, with a 43 residues shorter N-terminus. A third one lacking the complete exon 5 predicts a subunit without transmembrane segments. These forms, generated by alternative splicing, may account for the kinetic variability of the acetylcholine receptor channel.

Key words: Endplate channel; Muscle development; Splice variant

1. Introduction

The acetylcholine (ACh) receptor, one of the best known ion channels, exists in two forms, which are developmentally regulated. During fetal and early postnatal development, the skeletal muscle expresses the fetal form of the ACh receptor [1], composed of the $\alpha_2\beta\gamma\delta$-subunits. During this period important developmental changes occur, e.g. synapse elimination, and formation of the postsynaptic apparatus [2]. The γ-subunit confers on the receptor channel a long open time and low conductance, which produces slowly decaying postsynaptic currents. The kinetics of the fetal ACh receptor is highly variable. Measurements of closing rate [3], opening rate [4] and open time duration [5] have shown variable results. Single-channel conductance, on the other hand, shows a consistent value of 40 pS [3]. There is no explanation for the kinetic variability observed in native preparations.

The γ-subunit gene contains 12 exons, the first six encoding the N-terminus of the subunit [6]. Exons 7–9 and exon 12 encode the four transmembrane segment in a modular fashion. Large intronic sequences between exons 4–5 and 5–6 suggest the presence of modulatory elements.

The N-terminus of the α-subunit is responsible for the kinetic properties of the ACh receptor. It is possible that the N-termini of other subunits also participate in gating. The kinetic properties of the fetal type AChR channel have shown considerable variability, raising the possibility that the kinetic variability of the fetal channel results from the existence of different molecular forms with different N-termini and similar M2 conducting segments. Here we tested this hypothesis using the reverse transcriptase PCR reaction. The results demonstrate that, in addition to the described full-length subunit (γ1), three other forms are present in the muscle during early development. Two of these (γ2, γ4) encode a putative soluble peptide which may serve as a retrograde signal reporting successful synaptic contacts.

The splice variant form γ3 generates a complete subunit which could contribute to the ACh receptor channel diversity observed in muscle [3]. Channel events with similar conductance and several kinetic components can be explained by the presence of splice variants differing in the N-terminus. Since the conducting segment is probably the same, these forms are likely to produce a variability in the decay of synaptic currents.

2. Materials and methods

2.1. RT-PCR from muscle

Wistar rats were killed by decapitation and gastrocnemius muscles were dissected and quickly frozen under liquid nitrogen. Frozen muscles were ground in a mortar using a pestle, and total RNA was extracted using the RNeasy kit (Qiagen, Chatsworth, CA) following the specifications of the supplier. Reverse transcriptase reaction was carried out at 94°C in the presence of the reverse primer for 10 min using rTth polymerase (PE Biosystems, Mississauga, Ont.). Then the forward primer was added to run the PCR reaction. The PCR program consisted of an initial denaturation (2 min, 94°C) and 35 cycles (94°C, 30 s; 68°C, 30 s; 72°C, 30 s), followed by an extension period of 7 min at 72°C. We used as forward primers (see Fig. 1) 5′-AGCTCCTCTTGCTGCTGCTGGCTA (E1F), 5′-TCAGATGTGGTTAATGTCAG (E2F), and 5′-GAGGAGGCCCTCACGACTAATG (E5R). As reverse primers we used 5′-CAGGCGGCAGCCAGTAGATACA (E5R), 5′-TGGAGTCACAGGATCCAGTA (E7R), and 5′-AGCAGGTAGAACACCACCTTCT (E7fR).

Nested PCR, performed using Taq DNA polymerase (Gibco-BRL, Burlington, Ont.), produced a further amplification. The temperature protocol was similar to that of the RT-PCR reaction, except that the annealing temperature was optimized according to the melting temperature of the primers. Taq polymerase has the advantage of providing an extra adenine in the 3′ end, which is required for the TA cloning method [7].

PCR products were visualized in a 1.8% agarose gel stained with ethidium bromide. Bands containing the PCR fragments were excised from the gel using a scalpel and DNA was extracted using GeneClean (Bio101, Vista, CA). Individual bands were cloned into the pCR-2.1 vector from Invitrogen (Carlsbad, CA). In order to determine the proportion of the different subunit forms, 1 μl of the PCR reaction was directly ligated into the TA cloning vector.

3. Results

3.1. Multiple γ-subunit mRNAs are present in postnatal muscle

Since the single-channel conductance of the fetal ACh receptor channel is homogeneous, it seems plausible to attribute the kinetic variability to channels with the same conducting segment, and different N-termini. To test this possibility, we

*Fax: (1) (902) 494-1685.
E-mail: avillarr@is.dal.ca

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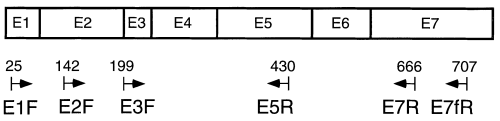

Fig. 1. Localization of primers used for RT-PCR amplification of the N-terminus region of the γ-subunit. The arrowheads indicate the 3′ end of the primers, and the numbers indicate the position of the first nucleotide. The starting codon ATG correspond to position 1 in the sequence.

amplified the region encoding the N-terminus using RT-PCR. Fig. 1 shows the set of primers used for PCR amplification together with the position along the sequence. RT-PCR reaction of RNA from P1 rat muscle, using primers E1F and E7fR (see Fig. 1), revealed a band whose length is consistent with the published sequence of the full-length γ-subunit. The expected size of this large fragment is 682 bp. The RT-PCR reaction, however, revealed in addition two additional bands with apparent molecular sizes of 583 and 319 bp (Fig. 2). Further amplification of the products of this reaction using nested primers E2F and E7R showed three bands with apparent molecular sizes of 506, 410, and 267 bp (Fig. 2B). Again, the largest band, which is also the most abundant, has a molecular size close to 525 bp, as predicted from the published sequence. The presence of two extra bands suggests that other mRNA forms containing sequences of the γ-subunits are present in muscle. Even though the use of nested primers reduces the possibility of a non-specific amplification, we ran further controls using the published γ-subunit DNA inserted into the pSPOoD vector [8]. The amplification of the pSPOoD-γ, using the nested primers E3F and E7R, revealed a single band, which indicates that the possibility of primer artifacts is unlikely (Fig. 2C). DNA amplified from muscle, on the other hand, reveal three bands. Comparable results were obtained with E2F-E7R nested primers (not shown).

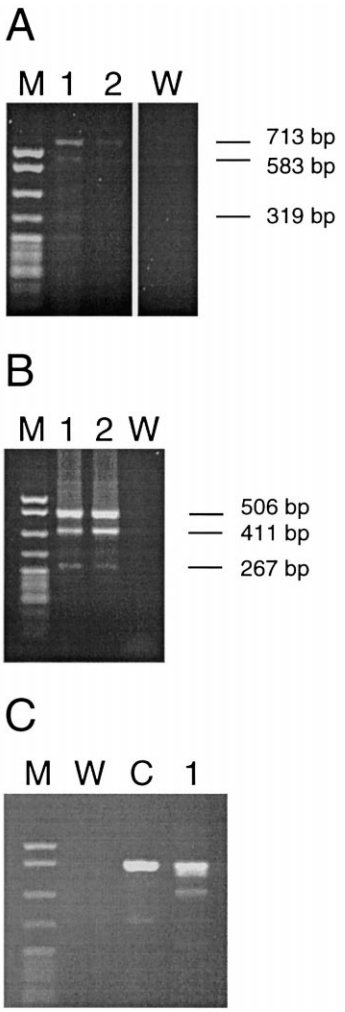

Fig. 2. RT-PCR amplification of the N-terminal region of the ACh receptor γ-subunit. A: RT-PCR products generated on RNA from P1 rat using the primers E1F and E7fR. Lanes 1 and 2 show RNA from a female and a male rat respectively; lane W shows control with no RNA. B: PCR products of the DNA from A, using nested primers E2F and E7R. C: Control PCR amplification using the pSPOoD-γ plasmid. Lane 1, DNA from RT-PCR from A; lane C, pSPOoD-γ plasmid. In both cases the nested primers E3F and E7R were used for amplification. W is a control with RNA replaced by water. In all cases, lanes M contain a pBR322 DNA digested with *Msp*I used as a molecular size marker.

3.2. *Alternative splicing generates differences in N-termini*

To clone the different molecular forms we followed two procedures. We excised a fragment of apparent molecular size 583 bp from a gel in which the complete 100 μl PCR mixture was run. DNA was extracted from the gel piece and cloned into the pCR-2.1 vector for further analysis. In addition, 1 μl of PCR mixture was directly ligated into the pCR-2.1 vector. With this procedure we obtained inserts of 525, 411, and 288 bp apparent molecular size.

We then analyzed restriction sites specific for each exon region. Exon 4 contains a unique site for *Eco*RV and exon 5 contains a unique site for *Hae*II. The pCR-2.1 plasmid contains a unique site for *Eco*RV and five sites for *Hae*II. Only the large size clones contained a site for *Eco*RV, indicating that a region within exon 4 was missing in the two others (not shown). Both the large and middle size clones carried a site for *Hae*II indicating that both carry sequences corresponding to exon 5.

Sequencing of seven clones corresponding to the 411 bp apparent molecular size reveals the absence of the whole region encoding exon 4. The absence of the exon 4 region alters the reading frame generating an opal stop codon. Such a sequence lacking exon 4 would translate into a mature peptide

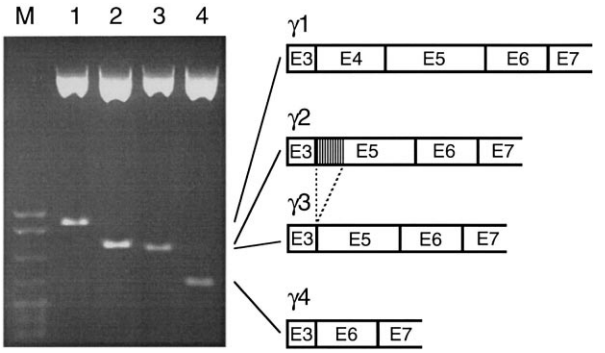

Fig. 3. Alternative splicing generates several N-terminal regions. The plasmid carrying the N-terminal sequence was digested with *Eco*RI, leaving 10 nucleotides from the vector. Sequence 1 corresponds to the complete subunit. Sequence 2 lacks exon 4. Sequence 3 lacks exon 4 and 19 bp of exon 5, maintaining the reading frame. Sequence 4 lacks both exon 4 and exon 5.

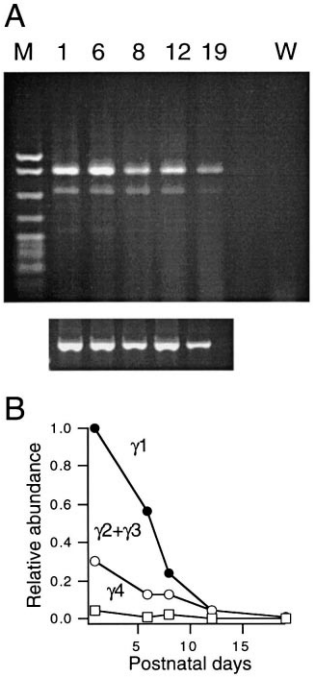

Fig. 4. Developmental change of the γ-subunit subtypes. A: RT-PCR of mRNA extracted from muscles of P1–P19 postnatal development using the E2F and E7R primers. The number indicates the postnatal day. W is a control with water. The insert at the bottom shows amplification of the corresponding RNAs using specific primers for β-actin. B: Quantification of three gels as in A. Each point corresponds to the average of the band intensity normalized to that of γ1 at postnatal day P1.

of 75 residues (see γ2 in Fig. 3). We also found clones which in addition lacked 19 bp from the 5′ end region of exon 5, which restores the reading frame. This sequence predicts a subunit lacking 34 amino acid residues within the N-terminus (see γ3 in Fig. 3). Sequencing of three clones corresponding to the apparent molecular size of 319 bp indicated that the complete sequence encoded in exons four and five was missing (see γ4 in Fig. 3). This form, similar to γ2, will also produce a short peptide of 89 residues, once the signal peptide is removed.

Analysis of 45 clones from RT-PCR amplification of RNA from four muscle samples according to their apparent molecular size revealed that 25 (55%) corresponded to the γ1 form. Fifteen γ2 clones accounted for 33% of the mRNAs. Equivalent proportions of γ3 (three clones) and γ4 (two clones) forms accounted for the remainder. This indicates that in addition to γ1, the most dominant form is γ2, which lacks transmembrane segments due to a stop codon. At least one of each of the γ1–γ4 forms was also found when we used E2F-E7R nested primers during the PCR amplification.

The new forms described here follow a time course similar to that of the γ1-subunit during muscle development. Fig. 4A indicates that γ1-mRNA, present in the muscle at day 1, becomes barely detectable by day 12. The γ2 and γ3 forms follow a similar time course, vanishing by day 19. The γ4-mRNA form is present at low levels throughout postnatal development.

4. Discussion

The results demonstrate that during early development skeletal muscle contains several mRNA forms encoding the AChR γ-subunit. Alternative splicing is the main mechanism generating such a diversity. Of the three new forms described, two (γ2, γ4) predict soluble peptides resulting from a stop codon. A third form (γ3) predicts a complete subunit peptide lacking part of the N-terminus. Based on the cloning data the most abundant form, after the complete γ1 form, is the putative soluble form γ2, which accounts for one third of all mRNA encoding the γ-subunits. The proportion of subunits is not likely to be a result of a cloning artifact since γ3, which differs from γ2 in only 19 bp, accounted for 6% of the clones. The possibility that larger sequences are easily incorporated into the pCR-2.1 vector seems unlikely.

4.1. Splice variant lacking exon 5 only was not found

A γ-subunit lacking exon 5 has been described in the C2C12 cell line and mouse muscle [9]. This form lacking exon 5 was present in newborn mice. The exon 5 sequence of the rat γ-subunit, when removed, preserves the reading frame producing a full subunit peptide. Such a form lacking exon 5, however, was absent in rat muscle. The only form lacking the complete exon 5 sequences was γ4, which also lacked exon 4 sequences. This may indicate differences in the RNA processing between mouse and rat muscle.

4.2. Developmental regulation

The subunit forms described here are developmentally regulated, as is γ1. By postnatal day 19 all of them become undetectable. The γ4 form is in low abundance throughout development, and it is difficult to describe its exact time course. This form becomes undetectable by day 12. Since the time course of all γ-subunit forms is similar, probably the expression of the pre-mRNA is regulated, rather than its processing. The disappearance of the γ-subunit mRNA forms described here using RT-PCR precedes that of the αβγδ receptor channels detected by patch-clamp [3]. Half of the functional receptor population becomes replaced by the αβεδ receptor by day 14 and they become undetectable by postnatal day 21.

4.3. Possible role of the short subunits

Two forms found (γ2, γ4) predict a soluble peptide corresponding to part of the extracellular portion of the N-terminus. It is possible that these peptides become processed and secreted. Secreted forms of membrane receptors, which arise from alternative splicing, are known in the antigen-related tyrosine phosphatase receptor [10]. If this is the case, one can speculate that these soluble peptides may act as a retrograde signal indicating that the γ-subunit gene is being transcribed. This signal could attract nerve endings eager to make synaptic contact with the muscle. Once the contact is established, and the ε-subunit is induced, no other nerve ending will be attracted. Upon denervation, the reappearance of γ-subunit expression guarantees re-innervation.

The putative secreted forms probably play a role in diseases such as myasthenia gravis. In some patients antibodies against the γ-subunit, which precipitate a soluble form of the human ACh receptor, have been found [11]. It will be of interest to know whether such forms correspond to the γ2 and γ4 forms described in this article. During early development when the immune system is not fully developed, this form will be unnoticed.

4.4. Role of the γ3 form

The γ3 form undergoes a processing that preserves the reading frame generating a complete subunit, presumably with all four transmembrane segments. This subunit, although it lacks 43 amino acid residues in the N-terminus, is likely to form a functional peptide. The three regions where the γ-subunit contacts the α-subunit, in the neighborhood of Lys-34, Ser-111 and Phe-172 [12], are preserved in this form. Lys-34, Ser-111 and Phe-172 are contained in exons 2, 5 and 6 respectively. It remains to be verified whether this subunit is able to generate functional receptors in an expression system such as *Xenopus* oocytes. It is possible that this subunit form contributes to the diversity of receptor channels observed in postnatal muscle.

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