Functional Changes Of Fetal Muscle Acetylcholine Receptor During Mouse Development

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In developing muscles in vivo and in vitro, the acetylcholine receptor γ-subunit exists in two splice variants, conferring different single-channel open durations (\(\tau_{op}\)) to reconstituted receptors. In mouse muscles, \(\tau_{op}\) changes around birth, possibly as receptors incorporate either variant of γ-subunit. This might be relevant to the concomitant maturation of muscle innervation.

The formation of the neuromuscular junction requires inductive interactions between nerve and muscle that lead to the coordinate development of specialized structures in both cells (as extensively reviewed in Ref. 5). The development of mature synapses from the first nerve-muscle contacts is remarkably slow; in rodents, it requires ~3 wk, the last (third) in utero and the first two after birth (1, 13). Among other changes, muscle innervation causes modifications in the distribution and structure of the acetylcholine receptor (AChR). Fetal muscle AChR is a pentameric protein assembled by \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\)-subunits, with the stoichiometry \(\alpha\beta\gamma\delta\). In mature neuromuscular junctions, an \(\epsilon\)-subunit replaces the \(\gamma\)-subunit. In mouse and rat, the \(\gamma\) to \(\epsilon\) switch takes place during the first 2 wk of postnatal life. At the end of fetal life, the mRNA encoding for γ-subunit is uniformly expressed over the entire fiber (8). After birth, γ-subunit mRNA tends to colocalize with the sites of innervation and gradually disappears by the end of the second week. The mRNA encoding for the \(\epsilon\)-subunit is first detected at birth, and it is present throughout adulthood (8), being invariably associated with subsynaptic nuclei. In close agreement, the sensitivity to ACh is distributed over the entire muscle fiber in young rats (2) but strictly limited to the junctional area in adult animals.

The structure of the AChR has been extensively reviewed (see Ref. 6, for instance), and it is only briefly summarized here. The five subunits forming the AChR are arranged around a central pore, which constitutes the ion permeation pathway. The γ-subunit is presumably located between the two α-subunits. Two non-equivalent ACh binding sites are present in each AChR, one formed by \(\alpha\)- and \(\gamma\)- or \(\epsilon\)-subunits and the other by \(\alpha\)- and \(\delta\)-subunits. The β-subunit does not contribute to the formation of agonist binding sites but is important in the formation of the full pentamer. The five AChR subunits have rather similar amino acid sequences within a species (33–47% in mouse), and the sequence of each subunit is well conserved across species (see Ref. 14). All the subunits have four transmembrane domains (M1 to M4), with M2 lining the pore (see Fig. 1) and an extracellular amino-terminal domain containing two cysteine residues (128 and 142) connected by a disulfide (S–S) bridge, which is characteristic of the receptor superfamily that comprises the receptors for glycine, γ-aminobutyric acid (type A), 5-hydroxytryptamine (type 3), as well as all the muscular and neuronal AChRs.

When the γ-subunit of the AChR is replaced by the \(\epsilon\)-subunit, unitary conductance and Ca\(^{2+}\) permeability of the receptor-associated ion channel increase, whereas channel open duration is reduced, as documented by a wealth of papers. These observations led to the notion that receptor structure determines its function. In addition to the subunit substitution mechanism, the developmental regulation of receptor structure, and hence function, may occur by means of alternative splicing of a common primary transcript. For example, all types of D-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) glutamate receptors in rodent brain exist in two alternative splice variants, which are differentially expressed before and after birth (12). Kinetic changes of the fetal form of AChR have been observed only for Xenopus laevis muscle in culture (9). No structural correlate of this modulation has been identified, and it is possible that splice variants underlie these changes.

In the mouse, the γ-subunit was cloned in the tumor B1H1 cell line (14). The existence of two RNA species of 2.1 and 1.95 kilobases was reported, although the origin of these two bands was not identified. Here, we describe the results

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of recent studies that characterized the two forms of $\gamma$-subunit mRNA, indicating that they arise by alternative splicing and showing that they are present in developing mouse muscles. The expression of these two splice variants might explain the modulation of the function of AChR channels observed around mouse birth.

**AChR $\gamma$-subunit has alternative spliced variants**

Using reverse transcription-polymerase chain reaction (RT-PCR), we identified and cloned in C$_2$C$_{12}$ mouse myotubes two mRNAs encoding for the AChR $\gamma$-subunit, one identical to that previously reported and a shorter variant missing the 52-amino acid domain encoded by exon 5 (short $\gamma$, $\gamma_s$) (11). This domain resides in the extracellular amino-terminal domain of the molecule and contains the disulfide-bonded cysteines and two residues likely to determine the selectivity in binding of competitive antagonists (reviewed in Ref. 6) (Fig. 1). The two mRNA variants coexist also in BC3H1 cells, as well as in muscle fibers from young or denervated adult mice (11). The $\gamma_s$-subunit can assemble into functional AChRs; this was demonstrated by injecting into *Xenopus* oocytes the mRNAs encoding for $\alpha$-, $\beta$-, and $\delta$-subunits plus either the $\gamma$-subunit ($\gamma$-AChR) or the $\gamma_s$-subunit ($\gamma_s$-AChR) mRNA (11). It is therefore conceivable that $\gamma_s$ plays a role in the in vivo development of mouse muscle fibers, where it is naturally expressed.

Lacking exactly one exon, $\gamma_s$ is likely to be a splice variant of the $\gamma$-subunit, which, in the mouse, is encoded by a single gene (14). To verify that the short mRNA variant was not artfactually produced by PCR amplification, we performed a ribonuclease protection assay on C$_2$C$_{12}$ cells. A riboprobe containing a region across exons 5 and 6 of the $\gamma$-subunit protected two bands, of 193 and 166 base pairs, corresponding to $\gamma$ and $\gamma_s$ variants, respectively (Fig. 2). The mRNA encoding for the $\gamma$-subunit was more abundant than the $\gamma_s$ transcript in all the four poly(A)$^+$ RNA preparations tested, confirming the

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**FIGURE 1.** Two variants of the muscle acetylcholine receptor (AChR) $\gamma$-subunit. A: schematic representation of the putative structure of mouse muscle $\gamma$-subunit. Like all the nicotinic receptor subunits, this structure has 4 membrane-spanning segments (M), with M2 lining the idrophilic pore. Exon 5 lies in the large extracellular amino-terminal domain and contains the disulfide-bridged cysteines (at positions 128–142), which is characteristic of the receptor superfamily, as indicated. B: nucleotide and deduced amino acid sequence of exon 5. Sequences are numbered as in Ref. 14. Deduced amino acid sequence is displayed above the corresponding nucleotide triplet. Exon 5 contains 156 base pairs (52 amino acids; indicated in boldface) and is removed when the primary transcript is spliced as short $\gamma$ ($\gamma_s$). Cysteines forming the disulfide-bonded loop are indicated by closed circles and a potential Asn-linked glycosylation site by an open circle.
results obtained by RT-PCR (4, 11). These data demonstrate that the mRNA lacking exon 5 is indeed produced by cell processing of the RNA encoding for the γ-subunit, yielding a splice variant of the subunit.

**Presence of γ-subunit variant increases channel open time**

Because the γ-subunit is a naturally occurring splice variant capable of assembling into functional receptors, at least in Xenopus oocytes (11), it was of interest to compare the functional properties of AChRs containing the γ- or the γ'-subunit variant. This study was performed in transiently transfected human BOSC 23 cells, using the patch-clamp technique to study ACh-evoked responses.

Although the expression level of γ'-AChR was lower than that of γ-AChR, ACh-evoked channel activity was recorded in cells transfected with either γ (n = 15 cells) or γ' (n = 10 cells) plus α-, β-, and δ-subunit cDNAs (3). For both γ- and γ'-AChRs, channel openings fell into a single amplitude class, corresponding to slope conductances of 36 and 34 pS, respectively (Fig. 3A). There was, however, a striking difference in channel opening kinetics, as the overall mean open time (τ_{op}) lengthened from 4 ms for the γ-AChR channel to 15 ms for the γ'-AChR channel (Fig. 3, A and B). The open time distribution was adequately fitted by two exponential components of almost equal weight for both AChRs (Fig. 3B). In the cells transfected with the γ'-subunit, receptors might contain only α-, β-, and δ-subunits, as several studies have shown that functional pentameric muscle AChRs might be composed of only three (but not less) subunit types. To demonstrate that the γ'-subunit is inserted into functional receptors, cells were transfected with α-, β-, and γ'-subunit cDNAs. ACh-evoked events showed a long τ_{op} (19 ms), whereas, in cells transfected with α-, β-, and δ-subunit cDNAs, τ_{op} was 5 ms. These data indicate that, at least in BOSC 23 cells, the γ'-subunit variant is included in AChRs, with a consequent prolongation of unitary channel openings.

In addition, the distributions of channel shut time differed between the two receptor species. For γ-AChR, the histogram of shut times was well fitted by the sum of three exponential functions, whereas an additional fourth component was required for an adequate fit of the shut time distributions of γ'-AChR channels, most likely reflecting the relatively low rate of openings observed in αβγδ-transfected cells.

**Channel open time is modulated during in vivo myogenesis**

Because both γ and γ' mRNAs are present in mouse muscles around birth and their relative expression appears to change perinatally (11), channel open time might be modulated by the incorporation of the γ'-subunit into functional membrane receptors. We therefore studied ACh-evoked unitary events in acutely dissociated muscle fibers obtained from mice aged between embryonic day 16 (E16) and postnatal day 6 (P6) (4). In all the fibers tested, channel openings had amplitudes corresponding to slope conductances of ~30 pS (Fig. 4A), typical of the fetal AChR. The τ_{op} of the ACh-evoked channels significantly increased from 6 to 9 ms between E16 and E19 and then decreased to 5 ms in the first postnatal week (Fig. 4). In patches with long τ_{op} events as long as 100 ms were observed. The histograms of open times were adequately fitted by two exponential components at all animal ages (Fig. 4, B and C). The fast component was remarkably constant during development, whereas the slow component closely followed the changes in τ_{op}, accounting for the existence of very long events at E18–E19 (Fig. 4C). When the same fiber was examined at two different places, τ_{op} varied by <1 ms (5 cells), whereas the rate of channel openings occasionally differed by as much as sevenfold.

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The histograms of closed times were adequately fitted by three components, but a detailed analysis of channel kinetics was not performed because in all patches more than one channel was active, as revealed by the presence of multiple openings. This implies that each $t_{\text{op}}$ was averaged over the several channels present in the patch so that the long openings observed at E18–E19 might be caused by an enhanced expression of $\gamma_s$-AChRs with respect to other ages. A weighted average of the open times measured in human BOSC 23-transfected cells shows that the $t_{\text{op}}$ values observed in dissociated fibers might be obtained if the fraction of $\gamma_s$-AChRs increased from 17% at E16 to 50% at E19 and decreased to ~10% in early postnatal life.

Conclusions

In developing muscle fibers in vivo and in vitro, we have identified and cloned a “short” variant of the muscle AChR $\gamma$-subunit (11), which is generated by alternative splicing of the primary transcript (4). The $\gamma$-subunit variant is capable of assembling into a functional AChR in heterologous systems, and it confers a peculiar long duration to ACh-evoked channel openings in transfected human BOSC 23 cells (3, 11). The expression of $\gamma$- and $\gamma_s$-AChRs might be modulated during mouse in vivo myogenesis, resulting in changes of the AChR channel open time, a phenomenon that was indeed observed in muscle fibers of mice between E16 and P6. This process is reminiscent of the developmentally regulated switch between alternatively spliced forms of a glutamate receptor (12).

FIGURE 3. Channel open duration is longer for short $\gamma$ ($\gamma_s$)-acetylcholine receptor (AChR) than for $\gamma$-AChR. All panels refer to cell-attached patches performed with an $\alpha$-$\beta$-$\gamma$-transfected cell (left, pipette potential of 0 mV) and an $\alpha$-$\beta$-$\gamma$-$\delta$-transfected cell (right, pipette potential of +20 mV), with 100 nM ACh in the patch pipette. A: typical examples of single-channel currents. Consecutive traces were digitally filtered at 1 kHz. In these 2 patches, channel conductance was 34.8 pS for $\gamma$-AChR and 34.2 pS for $\gamma_s$-AChR. Inward currents are represented by upward deflections. Note the very long channel openings in the $\alpha$-$\beta$-$\gamma$-$\delta$-transfected cell. B: histograms of channel open duration, fitted with the sum of 2 exponential functions (superimposed), with the indicated time constants, each having a weight of ~50%. Note the different time scales (abscissa). [Modified from Fucile et al. (3), copyright Blackwell Science, Osney Mead, Oxford, UK.]

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FIGURE 4. Channel open duration is modulated during in vivo myogenesis. Cell-attached patches were performed in acutely dissociated flexor digitorum brevis (fdb) muscle fibers from embryonic (E) or newborn mice (postnatal, P). Data shown in A–C were obtained in 1 patch for each animal age (as indicated). A: unitary currents were recorded with pipette potential set to 0 mV [embryonic day 16 (E16) and postnatal day 4 (P4)] or 30 mV (E19) to obtain an estimated transmembrane potential of about –80 mV. Acetylcholine amount was 100 nM. Slope conductances were 33.3 (E16), 31.6 (E19), and 29.2 (P4) pS. Inward currents are represented by upward deflections. Traces were filtered at 2 kHz. B: histograms of the channel duration. Mean open time ($t_{\text{op}}$) is indicated for each patch. Best fits are superimposed with the sum of 2 exponentials, with the following time constants: $t_1 = 0.5 \text{ ms} \ (18\%) \text{ and } t_2 = 6.8 \text{ ms} \ (82\%) \text{ at E16}; t_1 = 0.8 \text{ ms} \ (16\%) \text{ and } t_2 = 11.3 \text{ ms} \ (84\%) \text{ at E19}; t_1 = 0.9 \text{ ms} \ (32\%) \text{ and } t_2 = 4.2 \text{ ms} \ (68\%) \text{ at P4}.$ Note that different time scales were required to represent channel openings at each age. Best fits are superimposed with the sum of 2 exponentials, with the following time constants: $t_1 = 0.5 \text{ ms} \ (18\%) \text{ and } t_2 = 6.8 \text{ ms} \ (82\%) \text{ at E16}; t_1 = 0.8 \text{ ms} \ (16\%) \text{ and } t_2 = 11.3 \text{ ms} \ (84\%) \text{ at E19}; t_1 = 0.9 \text{ ms} \ (32\%) \text{ and } t_2 = 4.2 \text{ ms} \ (68\%) \text{ at P4}.$ Note that different time scales were required to represent channel openings at each age. C: time course of $t_{\text{op}}$ (•) and best-fitting time constants, $t_1$ (□) and $t_2$ (○), during mouse development. Values were averaged on 5–15 patches. $t_{\text{op}}$ at E19 is statistically different from values at E18 and P0 (P = 0.014 and P = 0.0004, respectively), all other values are not different from their neighbors. [Modified from Grassi et al. (4).]
The biphasic changes in $\tau_{op}$ are concomitant with, and might be relevant to, the maturation of nerve-muscle contacts into neuromuscular junctions (1, 13). The developmental significance of $\tau_{op}$ lengthening is emphasized by the observation that the increase of channel duration in hypothyroid mice is as retarded as the maturation of the neuromuscular junction in hypothyroid rats (4, 7). The establishment of nerve-muscle contacts induces many activity-mediated events in muscle fibers (reviewed in Ref. 5), and some of them, such as AChR clustering (10), require an increase in intracellular Ca$^{2+}$ concentration. It is tempting to speculate that $\gamma$-AChR channels control some of these processes, possibly because during prolonged openings more Ca$^{2+}$ enters into the cell.

The research from our laboratory was supported in part by Ministero dell’Università e della Ricerca Scientifica e Tecnologica grants to F. Grassi and F. Eusebi.

References