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Subunit-selective role of the M3 transmembrane domain of the nicotinic acetylcholine receptor in channel gating

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Abstract

The nicotinic acetylcholine receptor (AChR) can be either hetero-pentameric, composed of α and non-α subunits, or homo-pentameric, composed of α7 subunits. To explore the subunit-selective contributions of transmembrane domains to channel gating we analyzed single-channel activity of chimeric muscle AChRs. We exchanged M3 between α1 and ε or α7 subunits. The replacement of M3 in α1 by εM3 significantly alters activation properties. Channel activity appears as bursts of openings whose durations are 20-fold longer than those of wild-type AChRs. In contrast, 7-fold briefer openings are observed in AChRs containing the reverse ε chimeric subunit. The duration of the open state decreases with the increase in the number of α1M3 segments, indicating additive contributions of M3 of all subunits to channel closing. Each α1M3 segment decreases the energy barrier of the closing process by ~0.8 kcal/mol. Partial chimeric subunits show that small stretches of the M3 segment contribute additively to the open duration. The replacement of α1 sequence by α7 in M3 leads to 3-fold briefer openings whereas in M1 it leads to 10-fold prolonged openings, revealing that the subunit-selective role is unique to each transmembrane segment.

Keywords: Nicotinic receptor; Single-channel; Patch-clamp; Transmembrane domains

1. Introduction

The nicotinic acetylcholine receptor (AChR), member of the Cys-loop receptor superfamily, is of fundamental importance in the synaptic transmission at the neuromuscular junction and throughout the nervous system. AChR subunits are classified as α, which contain a disulphide bridge involved in the recognition and binding of agonists, and non-α subunits, which lack this motif. The primordial AChR presumably contained only one type of α subunit and evolution led to subunit diversity resulting in a wide spectrum of structurally and functionally different AChRs [1]. The AChR has a composition of $(\alpha1)_2\beta\delta\gamma$ in fetal and $(\alpha1)_2\beta\delta\varepsilon$ in adult muscle. In brain, receptors are made up either of different combinations of α (α2−α10) and β subunits (β2−β7) or of five identical α subunits, such as the neuronal α7 subtype. Each AChR subtype exhibits unique pharmacology and ion channel properties. Little is known about the selective role of each subunit during AChR activation.

AChR subunits share a similar structure: an amino-terminal extracellular domain, which includes the ACh binding sites, and a transmembrane region composed of four transmembrane segments (M1–M4). All four segments have been shown to contribute to channel function. The M2 domain delineates the ion pore, contributes to the cation selectivity and moves to allow ion flux [2–7]. The M1 domain appears to be involved in agonist dissociation and channel gating [8,9]. The M4 domain moves during channel activation and it may modulate AChR kinetics through its contacts with membrane lipids [10–14]. M3 also contributes to channel gating [15–17]. By tryptophan-scanning mutagenesis it has been postulated that M3 undergoes a spring motion during ion channel activation [18]. More recently, it was determined that M3 moves after M2 and M4 domains during the channel-opening process [19].

To further understand the role of the M3 transmembrane segment in each subunit of the pentameric receptor during channel activation we constructed chimeric subunits. We exchanged M3 between α and non-α subunits and between subunits that form homo- and hetero-pentameric receptors. To determine if the consequences of the transmembrane segment swapping are

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similar for different domains, we swapped α1M1 with M1 of ε and α7. Our results provide new evidence for the subunit-selective roles of M1 and M3 transmembrane domains and help to understand the differential contribution of subunits to channel activation.

2. Materials and methods

2.1. Construction of mutant and chimeric subunits and AChR expression

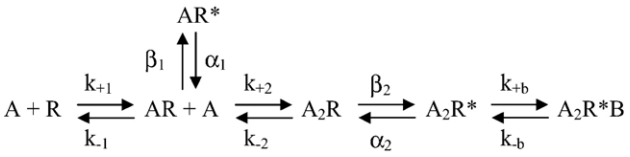
Chimeric and mutant subunits were constructed using the QuikChange™ Site-Directed mutagenesis kit (Strategene, Inc., TX). Restriction mapping and DNA sequencing confirmed all constructs.

HEK293 cells were transfected with mouse α1, β, δ, and ε cDNA subunits (wild-type or mutant) using calcium phosphate precipitation, essentially as described previously [20,21]. Cells were used for single-channel measurements 1 or 2 days after transfection. Surface expression of M3 chimeras was measured by determing the total number of [^{125}I]α-BTX binding sites. Transfected cells were incubated 2 h with 5 nM [^{125}I]α-BTX at room temperature. Non-specific binding was determined in the presence of 20 mM carbamylcholine. The total number of binding sites for all chimeric receptors varied between 3 and 10% of that of wild-type AChRs.

2.2. Patch-clamp recordings

Recordings were obtained in the cell-attached configuration [22] at a membrane potential of −70 mV and at 20 °C. The bath and pipette solutions contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$ and 10 mM HEPES (pH 7.4). Single-channel currents were recorded using an Axopatch 200 B patch-clamp amplifier (Molecular Devices Corporation), digitized at 5 μs intervals with the PCI-6111E interface (National Instruments, Austin, TX), and detected by the half-amplitude threshold criterion using the program TAC 4.0.10 (Bruxton Corporation) at a final bandwidth of 10 kHz [13]. Open- and closed-time histograms were fitted to the sum of exponential functions by maximum likelihood using the program TACFit (Bruxton Corporation).

Kinetic analysis was performed as described before [8,12,13]. The analysis was restricted to clusters of channel openings, each reflecting the activity of a single AChR. Clusters of openings corresponding to a single AChR were identified as a series of closely spaced events preceded and followed by closed intervals longer than a critical duration (τ_{crit}). This duration was taken as the point of intersection of the predominant closed component and the succeeding one in the closed-time histogram. The predominant closed duration component, which becomes shorter with the increase of agonist concentration, reflects the set of transitions between unliganded closed and diliganded open states. To minimize errors in assigning cluster boundaries, we analyzed only recordings from patches with low channel activity. Only clusters containing more than 10 openings and not showing double openings were considered for further analysis. For each recording, kinetic homogeneity was determined by selecting clusters on the basis of their distribution of mean open duration, mean closed duration and open probability [8,12,13]. Typically, more than 80% of the clusters were selected, and between 2000 and 10,000 events of each condition were used for the analysis. The resulting open and closed intervals from single patches at several ACh concentrations were analyzed according to kinetic schemes using QUB Software (QuB Suite, State University of New York, Buffalo). The dead time was typically 30 μs. Probability density functions of open and closed durations were calculated from the fitted rate constants and instrumentation dead time and superimposed on the experimental dwell time histogram as described by Qin et al. [23]. Calculated rates were accepted only if the resulting probability density functions correctly fitted the experimental open and closed duration histograms. Standard errors of the rate constants estimates were calculated by the program from the curvature of the likelihood surface at its maximum [23,24]. For wild-type AChRs the opening rate of the diliganded AChR, β_2 in Scheme 1, was constrained to its previously determined value [8,25,26] because brief closings due to gating and channel blocking become indistinguishable at high ACh concentrations [8,12,26]. Also, the association and dissociation rate constants were assumed to be equal at both binding sites [8,26,27].



Scheme 1.

Energy changes between different AChRs in channel closing were estimated as:

$$\Delta(\Delta Gc) = -RT \ln [(1/\text{mutant mean open time})/(1/\text{wild-type mean open time})] \quad (1)$$

where R is the gas constant and T is the absolute temperature. The closing rate was estimated by 1/mean open time. Open probability within clusters (P_{open}) was determined experimentally at each ACh concentration by calculating the mean fraction of time that the channel is open within a cluster.

For outside-out patch recordings, the pipette solution contained 134 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.3). Bath solution contained 150 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.3). A series of applications of bath solution containing 1 mM ACh were applied to the patch as described before [28,29]. Macroscopic currents were filtered at 5 kHz. Data analysis was performed using the IgorPro software (WaveMetrics Inc., Lake Oswego, Oregon). The ensemble mean current was calculated for 5–10 individual current traces. Mean currents were fitted by a single exponential function:

$$I_{(t)} = I_0 \exp(-t/\tau_d) + I_\infty \quad (2)$$

where I_0 and I_∞ are the peak and the steady state current values, respectively, and τ_d is the decay time constant that measures the current decay due to desensitization.

3. Results

3.1. M3 chimeric subunits assemble into functional receptors

To determine the specific role of the M3 segment of each subunit we constructed a series of chimeric receptors. We exchanged M3 between muscle α1 and ε subunits, and between α subunits forming hetero-(α1) or homo-oligomers (α7). We expressed the chimeric subunits with the complement wild-type subunits. Macroscopic currents recorded from outside-out patches rapidly perfused with 1 mM ACh reveal that the chimeric subunits are incorporated into functional receptors (Fig. 1). Currents are smaller than wild-type currents, probably due to the reduced cell surface expression, which is lower than 10% of that of wild-type AChR in all chimeric receptors (see Materials and methods). In contrast, the current decay time constants (τ_d) are similar to that of wild-type AChRs (τ_d=25±8 ms, 22±6, 27±7 and 25±3 ms for wild-type, α1(M3ε), ε(M3α1) and α1(M3α7), respectively).

Co-transfection of cells with chimeric and wild-type cDNA subunits could result in the surface expression of chimeric subunit-omitted AChRs. We can ensure that the chimeric subunits are incorporated into functional receptors because the absence of α1 cannot lead to functional receptors, and because channel activity recorded from cells transfected with α1, β, δ and the chimeric ε subunit is quite different to that of ε-lacking AChRs ($\alpha 1_2\beta\delta_2$) [20]. Considering the following issues, it is probable