Acetylcholine receptor subunit homomer formation requires compatibility between amino acid residues of the M1 and M2 transmembrane segments

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Abstract The neuronal nicotinic acetylcholine receptor (nAChR) subunits α3 and α7 have different assembly behavior when expressed in heterologous expression systems: α3 subunits require other subunits to assemble functional nAChRs, whereas α7 subunits can produce homomeric nAChRs. A previous analysis of α7/α3 chimeric constructs identified a domain comprising the first putative membrane-spanning segment, M1, as essential to homomeric assembly. The present study dissected further this domain, identifying three amino acid residues, which are located at the most intracellular third of the M1 transmembrane segment, as important in the assembly of homomers. Moreover, formation of homooligomeric complexes seems to require a compatible accommodation between this region and certain residues of the second transmembrane segment, M2. Thus, compatibility between defined domains of the M1 and M2 transmembrane segments appears as a determinant factor governing homomer association of nAChR subunits.

Key words: Nicotinic receptor; Subunit assembly; Transmembrane segment; Oligomer

1. Introduction

Neurotransmitter-gated receptors are oligomeric membrane proteins composed of homologous subunits (reviewed in [1,2]). Different subunit combinations yield different receptor subtypes with dissimilar channel properties (reviewed in [3]). The neuronal nicotinic acetylcholine receptor (nAChR) family is composed of agonist binding (α2-α9) and structural (β2-β4) subunits (reviewed in [4]). In heterologous expression systems it has been demonstrated that some α subunits (α2-α4) need to combine with structural subunits (β2, β4 and, in some cases, α5 [5,6]) to form heteromeric functional receptors. However, other α subunits (α7-α9) can form homomeric AChRs and do not seem to assemble with the ones which form heteromeric nAChRs [7-9]. The same situation may also occur in vivo, at least for some receptor subtypes. Thus, in chick ciliary ganglion cells [10,11], bovine chromaffin cells [12,13] and the human neuroblastoma SH-SY5Y [6] the α3 and α7 subunits seem to be components of two different receptor subtypes, which suggests that a mechanism may exist, in order to discriminate between these subunits, which are structurally similar.

In a previous analysis, we exploited the aforementioned differences between α3 and α7 subunits in multimer assembly to demonstrate that a domain, which includes the first putative transmembrane segment, M1, seems essential for homomer formation [14]. We have now extended further that study to show that three amino acids of the M1 segment, located close to its cytoplasmic end, determine the formation of homomers, probably as a result of compatibility or adaptability with some residues of the M2 transmembrane segment.

2. Materials and methods

2.1. Construction of chimeras and point mutants

Chimeras and point mutations were made essentially as described previously [15] by performing two successive PCR amplifications [14]. The mutated or chimeric DNA obtained from the second PCR was sequenced before being manipulated further with restriction enzymes, for insertion into its appropriate location in the original gene. All chimeras consist of the N-terminal region of the bovine α7 subunit [13], including a short 5' non-coding region, its leader peptide and part of the M1 membrane-spanning segment. The remaining regions of each chimera were composed of bovine α3 [12] or α7 subunit segments, as indicated in each figure legend.

2.2. Oocyte expression

Capped mRNA was synthesized in vitro using SP6 RNA polymerase (Boehringer Mannheim) and template DNAs inserted into pSP64T vector [16]. *Xenopus* (Nasco) oocytes were inoculated with 25 ng of RNA in 50 nl of sterile water. Characterization of the resulting nAChRs was performed between 3 and 4 days after injection.

The assembly capacity of each construct was assessed by measuring the total surface expression of α-bungarotoxin (α-Bgt)-binding nAChRs. Briefly, oocytes were preincubated at 18°C for 30 min in Barth's solution containing 5% fetal calf serum; then a saturating concentration (5 nM) of α-[^{125}I]Bgt (Amersham) was added, and the oocytes were incubated for 2 h at 18°C. The excess toxin was removed with five 4-ml washes of Barth's solution. Non-specific binding was determined using non-inoculated oocytes.

When chimeric constructs were able to produce functional nAChRs, oocyte membrane currents were measured with a two-microelectrode voltage-clamp amplifier as in [14]. This only applies to those constructs which contain the M2-M3 loop of α7 subunit (α7 mutants in Fig. 1 and the chimera C41 in Fig. 2), as we have previously shown that an Asp residue present in α7 (Asp^{266}) and β4 (Asp^{268}) but not in α3 subunits is essential to couple binding and gating in this type of nAChRs [17].

3. Results and discussion

The different assembly behavior shown by certain nAChR
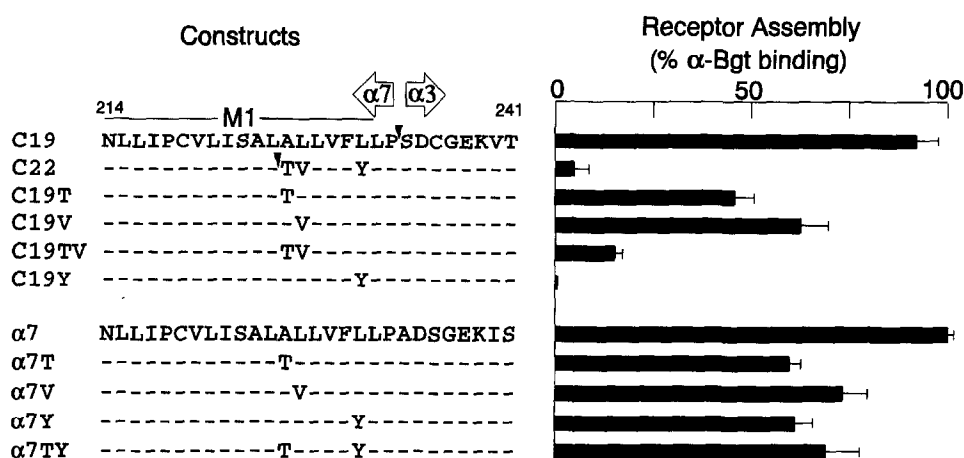
Fig. 1. Schematic diagrams and assembly capacity of α3/α7 constructs and α7 point mutants used to study the role of transmembrane segment M1 in homomeric assembly. The amino acid sequence of the M1 region of each chimera (designated C, top), or α7 mutant (α7, bottom) is displayed on the left. Arrowheads indicate the boundary between α7 (N-terminal) and α3 (C-terminal) sequences in the chimeras. Amino acid numbering relates to the α7 subunit [13]. Expression of the different constructs was tested by α-Bgt binding. All data were normalized to the mean obtained in the same oocyte batch with the α7 subunit alone, and the mean ± S.E.M. of at least 20 different oocytes from each of three different donors are shown for every construct. Typical values obtained with the α7 subunit were 3–5 fmol bound α-Bgt/oocyte. For the sake of brevity, construct C18, in which the α7 sequence extended up to Ile^{217}, has not been included in this figure. This construct did not give rise to assembled nAChRs.

subunits, such as the α3 and α7 polypeptides, which are expressed in the same cell but segregate to form different nAChR subtypes has been previously used to establish a model system in which to study the regions involved in intersubunit recognition [14]. Thus, α3/α7 chimeras were constructed and their cRNA injected into oocytes to produce homomeric nAChRs whose α-Bgt binding capacity was determined. This approach allowed us to reinforce and extend previous reports indicating the importance of the N-terminal region in intersubunit recognition [18–21], and to demonstrate that a short domain of 37 amino acids, which includes the first putative transmembrane segment M1, seems essential for homomer formation [14].

The present study dissected further this domain by constructing chimeras in which the α3/α7 boundary was located at positions intermediate between the previous ones shown to be able or not to produce homomeric nAChRs (designated C5 and C3, respectively, in [14]). Since in C3 the α7 sequence expanded from the N-terminus to Arg^{206} whereas in C5 it extended further to Leu^{242}, intermediate constructs were made, in which the α7 sequence extended up to Ile^{217} (C18), Leu^{225} (C22) and Pro^{233} (C19) (see scheme in Fig. 1). The first two constructs did not yield homomeric nAChRs whereas C19 produced α-Bgt binding activity close to that observed with α7 nAChRs (Fig. 1). As previously discussed [14], the strategy of measuring α-Bgt binding sites at the external oocyte membrane is expected to detect only properly assembled nAChRs that have followed an accurate maturation pathway from the endoplasmic reticulum to the oocyte plasma membrane [22]. In fact, solubilization and further sucrose gradient centrifugation of α-Bgt labeled nAChRs present in the external membrane of the oocytes yielded unique molecular species with a size close to that of a Torpedo nAChR composed of the α1$_2$β1γδ combination (not shown). Alternatively, the lack of α-Bgt binding could be due to the assembly of chimeric receptors unable to bind the toxin. This possibility appears unlikely if we consider that C19 and C22 differ in only three amino acids (Fig. 1) within an area located far away from

the toxin binding determinants and that the multiplicity of contacts between α-toxins and nAChRs results in a very strong interaction, which is difficult to distort. In fact, even denatured α subunit [23] or synthetic peptides (α186–196) [24] are able to bind α-Bgt. It is also important to note that we used α7 nAChR expression as an internal control in all experiments because of the inherent variability of the oocyte expression system: the results are expressed as a percentage of the α-Bgt binding observed with α7 nAChRs.

As mentioned above, only three amino acids (positions 226, 227 and 231) are different between C19 and C22, therefore they appeared responsible for the different behavior of these chimeras and were further mutated in C19 to make them appear as in C22. Constructs C19T (Ala^{226}Thr) and C19V (Leu^{227}Val) resulted in ~50% decrease in homomeric assembly (Fig. 1) whereas the double mutant C19TV (Ala^{226}Thr; Leu^{227}Val) yielded an even larger decrease (13% of the binding sites observed with α7 nAChRs). Finally, C19Y (Leu^{231}Tyr) was unable to produce homomeric nAChRs (Fig. 1). Thus, it was concluded that these three amino acids may play an important role in controlling homomeric assembly.

The chimeras used were composed of α7 (N-terminus) and α3 (C-terminus) sequences and it could happen that some of the effects observed in C19 mutants could not only be contributed by the mutated amino acids but also be due to a combined effect with some part of the α3 structure. Consequently, the same mutations were carried out in the wild α7 sequence and the assembly of the resultant mutants studied (Fig. 1, bottom). Two single mutants α7T (Ala^{226}Thr)and α7V (Leu^{227}Val) showed a slightly lower degree of assembly inhibition than their C19 counterparts. However, α7Y (Leu^{231}Tyr) only presented ~30% inhibition, in contrast with the total inhibition produced by C19Y. Even the double mutant α7TY (Ala^{226}Thr; Leu^{231}Tyr) did not show more than ~30–40% decrease in homomeric assembly. All α7 mutants produced functional responses upon activation with nicotine (not shown).
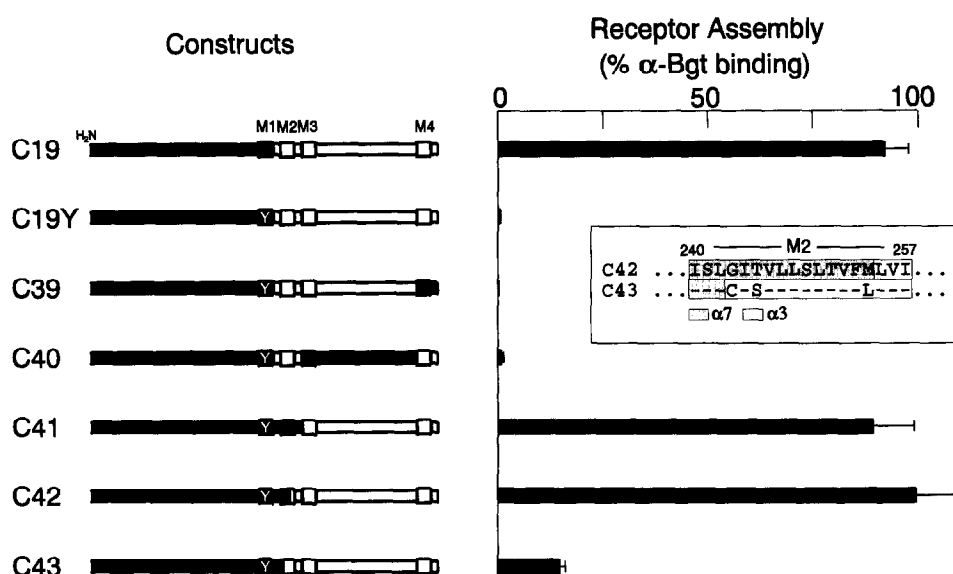
Constructs

Receptor Assembly
(% α-Bgt binding)

Fig. 2. Schematic diagrams and assembly capacity of α3/α7 constructs used to study the interaction of Leu/Tyr[231] with other parts of the α7 subunit. The structure of each chimera is displayed with empty and filled boxes representing α3 and α7 domains, respectively, whereas M1, M2, M3 and M4 are the membrane-spanning segments. A letter Y over M1 indicates that this construct contains the Leu[231]Tyr mutation. For comparison, C19 and C19Y results from the previous figure are included here. Assembly was tested as in Fig. 1. The inset represents the amino acid sequence of the M2 region of C42 and C43. Again, α3 and α7 sequences are indicated by open and shaded boxes, respectively. Amino acid numbering relates to the α7 subunit.

The results obtained with α7 mutants suggested that some domain, present in α7 but not in C19 and interacting with the most intracellular third of the M1 segment, was able to tolerate or accommodate the Leu[231]Tyr mutation, providing us with the opportunity of approaching its identification. The strategy followed was to construct chimeras similar to C19Y in which certain segments of the α3 subunit had been substituted by their α7 equivalent, thus trying to reverse the deleterious effect that the Leu[231]Tyr mutation had induced in C19. When segments corresponding approximately to transmembrane fragment M4 (Fig. 2, C39) or transmembrane fragment M3 and the cytoplasmic loop (Fig. 2, C40) were exchanged, no assembly of receptors was observed. By contrast, the incorporation of transmembrane fragment M2 and the loop linking it to M3 (i.e. when the α7 sequence continuously extended from the N-terminus to Gln[273]) restored receptor assembly (Fig. 2, C41) and functional responses (not shown). This domain was further analyzed by including only part of transmembrane fragment M2. When the α7 sequence reached up to Met[254] nAChRs were expressed (Fig. 2, C42), while a shorter extension, up to Leu[242], produced a lack of assembly (Fig. 2, C43). Only three amino acids are distinct when comparing C42 and C43 (see inset in Fig. 2) and, therefore, one or several of these residues would conform to the area which interacts with Leu/Tyr[231]. As this residue should be close to the cytoplasmic side of the membrane, whatever the structure assumed for the M1 membrane-spanning segment, it is reasonable to infer that amino acids Gly[243] and/or Thr[245] in M2 are the ones interacting with the former, since they are more cytoplasmically located than Met[254].

The results obtained in the present report support the idea that, in addition to N-terminal domains [14,18–21,25,26], certain amino acids in the M1 transmembrane fragment control α7 subunit homomeric assembly. Moreover, at least for Leu/Tyr[231], this control seems to be produced by way of its inter-

action with other amino acids from the M2 segment. As in the case of the N-terminal domains mentioned above, the nature of this interaction is not obvious upon inspecting the amino acids involved. Thus, it is tempting to invoke some sort of steric complementarity between amino acid chains which, given their location in the hydrophobic membrane-spanning segments, might be tightly packed. Consequently, only compatible residues would allow the assembly of nAChRs.

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