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A small cytoplasmic region adjacent to the fourth transmembrane segment of the α7 nicotinic receptor is essential for its biogenesis

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ABSTRACT

Deletion of a small cytoplasmic fragment close to the fourth transmembrane segment of the nicotinic α7 receptor (Glu437 to Arg447) abolished membrane expression. Different single mutants showed moderate to strong decreases in expression whereas the latter was totally abolished upon proline substitutions. We hypothesize that preservation of an α-helix formed by the fourth transmembrane segment and the adjacent cytoplasmic region is essential for membrane receptor expression. Moreover, in selected mutants with low or null membrane expression, a significant proportion of mature receptors was present inside the cell. Hence, elements in this cytoplasmic fragment might influence receptor transport to the membrane.

1. Introduction

Nicotinic acetylcholine receptors (nAChR) are members of a supergene family of ligand-gated ion channels [1]. These membrane proteins are pentameric oligomers composed of five homologous subunits surrounding the ion pore [2]. Each subunit is composed of ~500 amino acids and four transmembrane segments. A large extracellular region at the N-terminus is well conserved among subunits and contains important elements for agonist binding [3,4] and channel gating [4,5]. The other large region is a cytoplasmic loop located between the third (M3) and fourth (M4) transmembrane segments and is variable in amino acid sequence and length. Included in this loop there is a putative amphipatic helix (MA), which is close to M4 [6]. Previously, we have shown that deletion of the small linker between M4 and MA totally abolished membrane expression [7]. For this reason, we have carried out a more detailed study of this linker, showing the strong influence of some amino acids on receptor biogenesis and the probable need of a continuous α-helix in this region for proper membrane expression.

2. Materials and methods

2.1. Generation of mutants of the bovine α7 subunit

The bovine α7 cDNA was cloned in a derivative of the pSP64T vector [8] containing part of the pBluescript polylinker. Mutants were generated by using single-stranded oligonucleotides with the desired sequences and proper single-strand ends which could be easily ligated to the ends generated by restriction enzymes either present in the original sequences or introduced by PCR as silent mutations.

2.2. Oocyte expression

Capped mRNA was synthesized in vitro using SP6 RNA polymerase with the mMESSAGE–mMACHINE kit (Applied Biosystems, Madrid, Spain). Defoliculated *Xenopus laevis* oocytes were injected with 550 μg/μl of total cRNA in 50 nl of sterile water. Oocytes were incubated in calcium-free medium. All experiments were performed within 2 days after cRNA injection. mRNA of wild-type (WT) receptors was injected into oocytes from the same frog every time a mutant was tested. Consequently, mutant expression was expressed as a percentage of WT receptor expression observed in the same experiment.

2.3. [^{125}I]-α-bungarotoxin binding assays

Specific surface expression of [^{125}I]-α-bungarotoxin (α-Bgt) (PerkinElmer España, Madrid, Spain) binding sites was tested with

Abbreviations: ACh, acetylcholine; α-Bgt, α-bungarotoxin; amphipatic helix, MA; fourth transmembrane segment, M4; nAChR, nicotinic acetylcholine receptor; WT, wild-type

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12 nM [^{125}I]-α-Bgt exactly as described [9]. Given the location of the mutations, it is unlikely that they can affect α-Bgt binding properties. Nevertheless, some low-expressing mutants were analyzed with higher toxin concentrations to discard potential decreases in toxin affinity.

Total [^{125}I]-α-Bgt binding sites were obtained by carrying out the incubations in the presence of 0.1% saponin.

2.4. Electrophysiological recordings

Electrophysiological recordings were carried out as previously described [10]. Functional expression of each construct was estimated as the peak ionic current evoked by 1-s application of 1 mM ACh at –80 mV and no correction for desensitization was made. All experiments were performed at room temperature (22 °C).

2.5. Western blot

Oocytes were injected with RNAs of α7 WT or mutant subunits containing the FLAG epitope repeated three times. This allowed a very specific and efficient detection of expressed receptors. The exact amino acid sequence added to the C-terminus of each subunit was: TVQGDYKDHDGDYKDHDIDYKDHD and did not affect the results previously observed with WT and mutant subunits. Total membranes of injected oocytes were prepared by a modification of a previously described procedure [10]. Eight oocytes were homogenized in 1.4 ml of Barth's buffer supplemented with a protease inhibitor cocktail (product 04 693 124 001; Complete from Roche, Barcelona, Spain) used as suggested by the manufacturer. Homogenates were centrifuged at 250×*g* for 10 min at 4 °C to discard cell debris and the supernatant was centrifuged at 16 000×*g* for 20 min at 4 °C to pellet down total membranes. Pellets were resuspended in 1.4 ml of the same buffer and centrifuged again in the same conditions. Pellets were resuspended in 0.2 ml of the same buffer, aliquoted and frozen until use. Before electrophoresis, membranes were dissolved in Laemmli's sample buffer and proteins were separated by 10% dodecyl sulfate-polyacrylamide gel electrophoresis. After the transfer, nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) were blocked 90 min at 22 °C with 5% dry milk in phosphate-buffered saline–0.05% Tween 20, and incubated overnight at 4 °C with anti-FLAG antibody M2 (Sigma, 1:4000 dilution in phosphate-buffered saline–0.05% Tween20 and 1% dry milk). Membranes were further treated as previously described [9].

3. Results

3.1. An alanine scanning mutagenesis study of the linker between the fourth transmembrane domain and the amphipathic helix of the α7 nAChR

The linker between MA and M4 does not seen to be structured (see Fig. 1B for a ribbon diagram of a single subunit of the bovine α7 nAChR homologous to the Torpedo nAChR subunits shown aligned in Fig. 1A [6]). Since no expression of receptors was observed upon its deletion [7] we decided to analyze single mutants of this region. Each amino acid, from Glu437 to Arg447 was replaced by alanine (except Ala441 and Ala442, see Fig. 2, upper panel). Expression of nAChRs was monitored by measuring α-Bgt binding sites at the external surface of oocytes. Typical values obtained with the bovine α7 subunit were 5 fmol of bound α-Bgt/oocyte and 4 μA/oocyte at −80 mV. Surface receptor expression was almost abolished in mutants V444A and D446A (Fig. 2). The requirements for these residues at their positions were different: Asp446 did not admit the conservative change to glutamate whereas the moderate exchange of Val444 for isoleucine restored receptor expression (Fig. 2). Moderate decreases in expression were observed for W438A, K439A, F440A and V445A, whereas expression of E437A and C443A was not affected. Mutants of Arg447 showed a variety of effects, difficult to correlate. Thus, R447A showed more than 3-fold increase in expression but other hydrophobic substitutions like R447M and R447V were very poorly expressed. The same happened with the polar change to serine. Finally, the conservative change to lysine restored expression to almost normal levels.

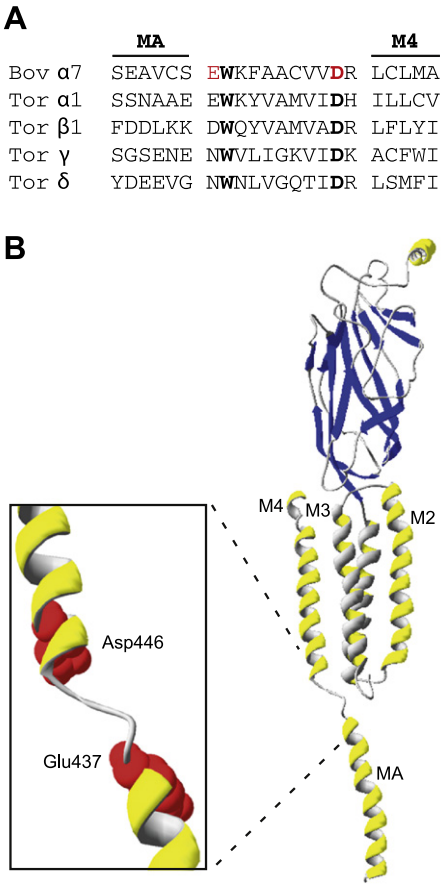

Fig. 1. (A) Sequence alignment of the region between MA and M4 of the bovine α7 (including small portions of the latter, from Ser431 to Ala452) and the *Torpedo* nAChR subunits. In bold are indicated the conserved amino acids. In red amino acids enlarged at the inset below. (B) Overall layout of a nAChR subunit showing the extracellular and transmembrane regions. Shown is a subunit from a homology model of the bovine α7 nAChR. Domains with α-helical structure are shown in yellow whereas the extracellular region made of β-sheets is shown in blue. The region between M4 and MA is enlarged at the inset to show in red the two negatively charged amino acids (Glu437 and Asp446) that flank the region of interest in this study.

Overall current amplitudes were not affected in the mutants, being their values with respect to control receptors similar to the ones observed in the α-Bgt binding assays (Fig. 2, black boxes).

3.2. Proline substitutions abolished membrane receptor expression

The linker between MA and M4 contains two alanines at its center (Ala 441 and Ala442). Given the high propensity of this amino acid to maintain α-helices [11] we speculated that a continuous α-helix would form all along the elements mentioned above. In order to break this potential α-helix, we decided to replace the alanines by prolines. Surface receptor expression of mutants A441P and