FEBS Letters 585 (2011) 2477–2480

FEBS Letters

journal homepage: www.FEBSLetters.org

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

Manuel Criado *, José Mulet, Susana Gerber, Salvador Sala, Francisco Sala

Instituto de Neurociencias, Universidad Miguel Hernández-CSIC, Sant Joan d'Alacant, 03550 Alicante, Spain

ARTICLE INFO

Article history:
Received 6 June 2011
Revised 21 June 2011
Accepted 22 June 2011
Available online 1 July 2011

Edited by Maurice Montal

Keywords:
Nicotinic receptor
Cytoplasmic domain
α-Helix
Maturation
Membrane transport

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

* Corresponding author.
E-mail address: manuel.criado@umh.es (M. Criado).

0014-5793/$36.00
doi:10.1016/j.febslet.2011.06.028

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.

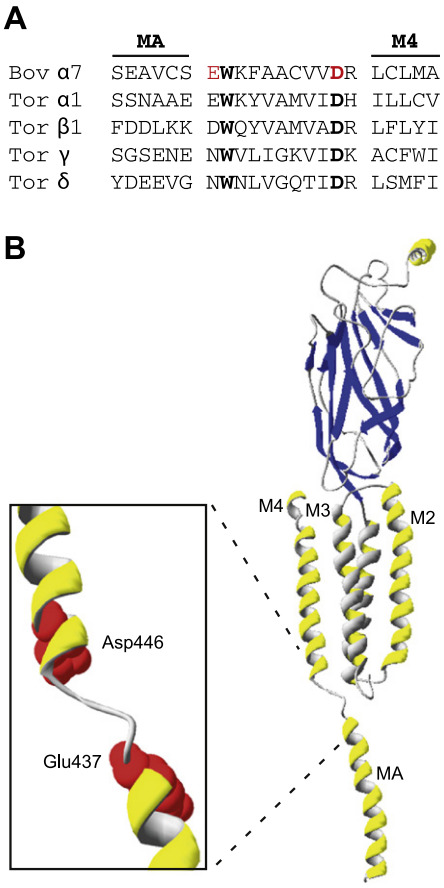

Fig. 1. (A) Sequence alignment of the region between MA and M4 of the bovine α7 (including small portions of the latter, from Ser431 to Ala452) and the *Torpedo* nAChR subunits. In bold are indicated the conserved amino acids. In red amino acids enlarged at the inset below. (B) Overall layout of a nAChR 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

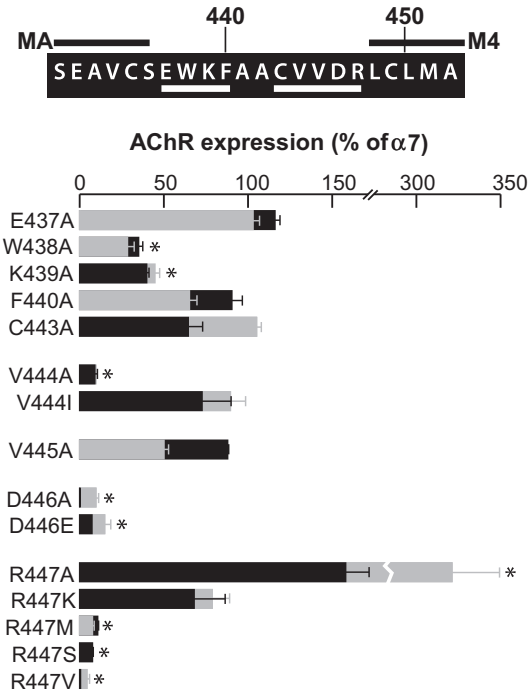

Fig. 2. Effect of single alanine substitutions at the linker between MA and M4 of the α7 nAChR on receptor expression. The sequence at the top shows the region of interest including the ends of MA and M4 segments. The amino acids mutated to alanine are underlined. All amino acids between Glu437 and Arg447 were mutated to alanine and surface expression of mutant receptors was tested by α-Bgt binding (open bars). Functional responses are also shown (black bars). Open and black bars are superimposed to simplify the figure. Those residues whose mutants yielded low expression were further mutated. All data were normalized to those obtained with WT α7 nAChRs and mean ± S.D. of data obtained from two-four batches of oocytes (50 oocytes in each batch for binding and 8 oocytes for currents) are shown. Asterisks represent statistically significant differences (both in binding and currents data) with respect to WT α7 ($P < 0.01$), that were calculated by one-way ANOVA test and by Bonferroni's multiple comparison test.

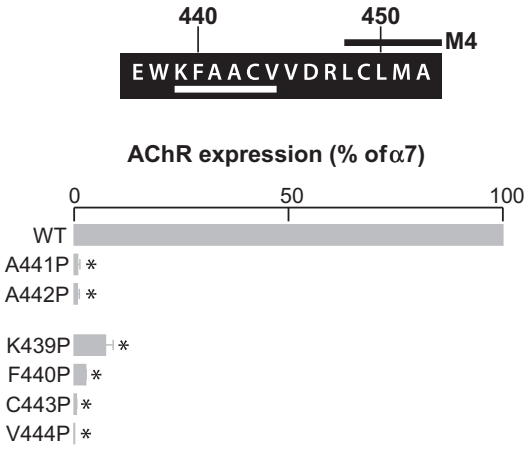

Fig. 3. Effect of single proline substitutions at the linker between MA and M4 of the α7 nAChR on receptor expression. All amino acids between Lys439 and Val444 (see the underlined sequence at the top) were mutated to proline and surface expression of mutant receptors was tested by α-Bgt binding to oocytes. Experimental conditions as in Fig. 2.

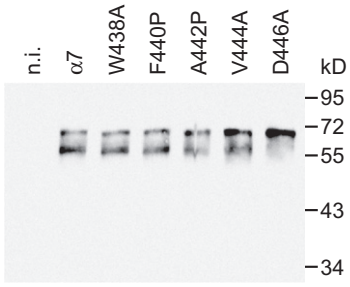

Fig. 4. Expression of different α7 mutants in oocytes. Total membranes of *Xenopus* oocytes were dissolved and an equal amount of protein separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with an anti-FLAG antibody. Molecular weight marker (in kDa) is indicated at the right. Two more batches of oocytes yielded similar results.

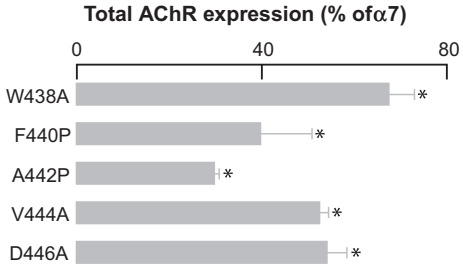

Fig. 5. Total expression of different α7 mutants in oocytes. The total amount of nAChRs was determined with α-Bgt in the presence of 0.1% saponin and is expressed as percentage of the total expression measured for α7 WT receptors. Experimental conditions as in Fig. 2.

A442P was almost abolished (Fig. 3). To discard that the breaking effect of the inserted prolines was position-dependent we also mutated two other residues at both sides of Ala441 (K439P and F440P) and Ala442 (C443P and V444P). Again, surface expression was strongly reduced, so that the highest expression was observed for K439P (7.3% of WT α7 receptor, Fig. 3). Currents evoked by ACh in these mutants also decreased in a parallel way (not shown).

3.3. Low surface receptor expression is not due to decreased steady-state levels of receptor subunits

We asked whether mutants that were expressed at the oocyte membrane at low levels were retained inside in a relatively stable way or were rapidly degraded. For this purpose, we compared the steady state levels of wild-type and some representative mutant receptors such as W438A, F440P, A442P, V444A and D446A. They showed 28.4%, 2.7%, 0.7%, 9.7% and 9.6% of the surface expression observed in WT receptors, respectively. A western blot of oocyte total membranes indicated that the amounts of WT and mutant subunits were similar (Fig. 4), suggesting that, independently of their surface membrane expression levels, mutant subunits were as stable as WT ones and, therefore, their low membrane expression is not a consequence of reduced subunit synthesis or accelerated degradation. Two very close bands were observed, probably indicating the existence of subunits with different carbohydrate content, as we have previously shown for WT α7 and mutants of the N-terminal α-helix [9]. An exception was mutant D446A, in which the larger band was predominant, suggesting that in this case most of the subunit pool was equally glycosilated.

3.4. A significant proportion of mutant subunits remained inside the oocytes as mature receptors

The presence of saponin during the binding experiments allowed the entry of α-Bgt to the oocyte. In this way, we could know whether mutant subunits retained inside were able to form receptors which have matured enough to bind the toxin. We tested the

same mutants having low or null membrane expression, but whose total expression was similar to the WT receptor, as shown by western blots (see Fig. 4). All mutants exhibited a significant proportion of total α-Bgt binding sites, ranging from a 30% of WT receptor for A442P to almost 68% for W438A (Fig. 5). Therefore, mutant subunits are able to assemble mature receptors but many of them do not reach the oocyte membrane.

4. Discussion

Biogenesis of nAChRs is a complex process [12] in which different extracellular and cytoplasmic as well as transmembrane domains of nAChRs appear to be involved [13]. Deletion of the intracellular fragment that connects MA and M4 of the α7 nAChR abolished membrane receptor expression [7], suggesting its potential relevance during receptor biogenesis. We hypothesized that this linker could be a key folding domain serving as bridge between the transmembrane region, through M4, and the whole intracellular region, through MA. Actually, single substitution of residues from Glu437 to Arg447 indicated that several of them were needed for proper expression of α7 nAChRs at the oocyte membrane (Fig. 2). This was particularly noticeable for Val444, Asp446 and Arg447, which are conserved in all neuronal nicotinic subunits. In the case of Val444 and Arg447, similar amino acids could substitute them. However, the requirement for Asp446 was tighter, as even glutamate could not substitute it. This aspartate residue is also much conserved in other members of the Cys-loop superfamily of receptors and, in fact, its mutation in the α1, β2 and γ2 subunits of the $GABA_A$ receptor caused reduced formation of pentameric receptors and retention in the endoplasmic reticulum [14].

Despite the strong decrease on membrane receptor expression, the total subunit levels of representative mutants such as W438A, F440P, A442P, V444A and D446A were similar to the one of the WT α7 nAChR (Fig. 4). This situation was also observed with several mutants of the N-terminal α-helix [9] and the loop between β-strands β2 and β3 [15], that were as stable as WT subunits. However, in the latter the capacity to oligomerize was strongly hindered, whereas in the present study, mutant subunits were able to assemble receptors mature enough to bind α-Bgt, although at a lower extent than WT receptors. Therefore, the region studied here seems to be needed for the correct assembly of mature receptors and, in addition, is especially important for their transport to the oocyte plasma membrane.

Structural information about the studied region is limited to the electron microscopy data of Unwin [6]. The region appears as a poorly structured linker between the MA and M4 α-helices. Proline residues were introduced at different locations of this linker inducing strong decreases in membrane expression (Fig. 3). A possible explanation for this result could be that the presence of a proline would disrupt a continuous α-helix along MA and M4. This α-helix would be needed for proper folding and, therefore, transport to the membrane. This hypothesis would be supported by the presence, at the middle of the linker, of two alanines, which tend to favor the formation of α-helices [11]. Conversely, we could assume that the linker is not structured and that a certain flexibility, needed during receptor biogenesis, is lost upon proline insertion. Whatever the case, the present results suggest that the studied region play an essential role in receptor biogenesis, probably by acting as a bridge that maintains adequately connected the transmembrane and intracellular regions of the α7 nAChR.

Note added in proof

In a recent paper (Nature doi:10.1038/nature10139; vol. 474, pages 54–60) Hibbs and Gouaux have shown the three-dimensional structure of an anion-selective Cys-loop receptor at 3.3 Å resolution. Although they deleted a large part of the cytoplasmic loop that included MA, the region that we studied here is still present in their receptor and shown to form a continuous, slightly bended, α-helix with M4. Thus, our hypothesis about the structure of this region seems to be supported by crystallographical data obtained in a homologous receptor.

Acknowledgements

This work was supported by grants BFU2008-02160 and CSD2008-00005 (The Spanish Ion Channel Initiative-CONSOLIDER INGENIO 2010) from the Ministry of Science and Innovation of Spain. We thank Stuart Ingham for the graphics work.

References

[1] Albuquerque, E.X., Pereira, E.F.R., Alkondon, M. and Rogers, S.W. (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol. Rev. 89, 73–109.
[2] Absalom, N.L., Schofield, P.R. and Lewis, T.M. (2009) Pore structure of the Cys-loop ligand-gated ion channels. Neurochem. Res. 34, 1805–1815.
[3] Rucktooa, P., Smit, A.B. and Sixma, T.K. (2009) Insight in nAChR subtype selectivity from AChBP crystal structures. Biochem. Pharmacol. 78, 777–787.
[4] Miller, P.S. and Smart, T.G. (2010) Binding, activation and modulation of Cys-loop receptors. Trends Pharmacol. Sci. 31, 161–174.
[5] Cederholm, J.M.E., Schofield, P.R. and Lewis, T.M. (2009) Gating mechanisms in Cys-loop receptors. Eur. J. Biophys. 39, 37–49.
[6] Unwin, N. (2005) Refined structure of the nicotinic acetylcholine receptor at 4A resolution. J. Mol. Biol. 346, 967–989.
[7] Valor, L.M., Mulet, J., Sala, F., Sala, S., Ballesta, J.J. and Criado, M. (2002) Role of the large cytoplasmic loop of the α7 neuronal nicotinic acetylcholine receptor subunit in receptor expression and function. Biochemistry 41, 7931–7938.
[8] Krieg, P.A. and Melton, D.A. (1984) Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res. 12, 7057–7070.
[9] Castillo, M., Mulet, J., Aldea, M., Gerber, S., Sala, S., Sala, F. and Criado, M. (2009) Role of the N-terminal α-helix in biogenesis of α7 nicotinic receptors. J. Neurochem. 108, 1399–1409.
[10] Leduc-Nadeau, A., Lahjouji, K., Bissonnette, P., Lapointe, J.-Y. and Bichet, D.G. (2007) Elaboration of a novel technique por purification of plasma membranes from Xenopus laevis oocytes. Am. J. Physiol. Cell Physiol. 292, 1132–1136.
[11] Blaber, M., Zhang, X.J. and Matthews, B.W. (1993) Structural basis of amino acid alpha helix propensity. Science 260, 1637–1640.
[12] Millar, N.S. and Harkness, P.C. (2008) Assembly and trafficking of nicotinic acetylcholine receptors. Mol. Membr. Biol. 25, 279–292.
[13] Tsetlin, V., Kuzmin, D. and Kasheverov, I. (2011) Assembly of nicotinic and other Cys-loop receptors. J. Neurochem. 116, 734–741.
[14] Lo, W., Botzolakis, E.J., Tang, X. and Macdonald, R.L. (2008) A conserved Cys-loop receptor aspartate residue in the M3–M4 cytoplasmic loop is required for GABAA receptor assembly. J. Biol. Chem. 283, 29740–29752.
[15] Criado, M., Mulet, J., Castillo, M., Gerber, S., Sala, S. and Sala, F. (2010) The loop between β-strands β2 and β3 and its interaction with the N-terminal α-helix is essential for biogenesis of α7 nicotinic receptors. J. Neurochem. 112, 103–111.