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An extracellular RRR motif flanking the M1 transmembrane domain governs the biogenesis of homomeric neuronal nicotinic acetylcholine receptors

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ABSTRACT

We have previously demonstrated that the highly conserved R209, that flanks the M1 transmembrane segment of nicotinic acetylcholine (ACh) receptors, is required for the transport of assembled homomeric neuronal α7 nicotinic ACh receptors to the cell surface. In the present paper we show that basic residues at positions 208 and 210 are necessary for the assembly of α7 receptors. On the contrary, a basic residue at position 210 of α3 subunit decreases the assembly of heteromeric neuronal α3β4 nicotinic ACh receptors. A basic residue at position 210 of the β4 subunit slightly decreases α3β4 receptor expression. We conclude that a pre-M1 RRR motif is necessary for the biogenesis of homomeric α-bungarotoxin-sensitive neuronal α7 nicotinic ACh receptors.

1. Introduction

The Cys-loop superfamily of ligand-gated ion channels (LGIC) constitutes a gene superfamily that includes the nicotinic acetylcholine receptor (nAChR), the glycine receptor, the 5-HT_3 receptor and the $GABA_A$ and $GABA_C$ receptors [1,2]. It has been clearly established that the members of this superfamily have a remarkable degree of identity at their primary structure level, including several residues conserved in all subunits of all receptors, e.g., cysteine 128, cysteine 142 or arginine 209.[2] It is also accepted that the LGICs share a similar secondary structure: a long extracellular N-terminal agonist-binding domain followed by four predicted transmembrane segments (M1, M2, M3 and M4) and a short extracellular C-terminal region. Between M3 and M4 there is a cytoplasmic loop that often contains putative regulatory sites of phosphorylation [1,2]. In response to specific agonists, LGIC regulate the opening and closing of an ion channel -that is an integral part of their structures- inducing a depolarization or hyperpolarization of the membrane, depending on the ionic selectivity of the channel. nAChRs are the best characterized members of LGIC. Muscle-type nAChRs are pentamers composed of four different subunits with the subunit composition $(\alpha1)_2(\beta1)(\delta(\gamma \text{ or } \varepsilon)$ that bind the snake venom neurotoxin α-bungarotoxin (Bgtx). Neuronal-type nAChRs that do not bind α-Bgtx are formed from combinations of either α2, α3, α4 or α6 agonist-binding subunits along with β2, β3, β4 and/or α5 structural subunits. They usually assemble with a stoichiometry of $(\alpha)_2(\beta)_3$, although they can assemble with an alternative stoichiometry of $(\alpha)_3(\beta)_4$. There is also evidence for neuronal nAChRs containing three and four different subunits. α7 and α8 subunits form homopentamers that bind α-Bgtx, although a minor population of nAChRs containing both α7 and α8 subunits has been isolated [3]. No structural subunits for this kind of neuronal nAChRs have been described [4,5]. As for α7 and α8, the α9 subunit is able to form homomers that bind α-Bgtx, but it is usally co-assembled with α10 subunits in native nAChRs [3]. α10 subunit is unable to evoke any current when expressed alone [6].

R209 is an invariant residue flanking the M1 transmembrane segment (Fig. 1). We have previously demonstrated that R209 is required for the transport of assembled receptors to the cell surface in αBgtx-sensitive neuronal α7 nAChRs. By contrast, R209 does not play any role in the transport of assembled αBgtx-insensitive neuronal α3β4 nAChRs to the cell surface [7]. However, a basic residue at this position of the α3 subunit is neces-

Abbreviations: ACh, acetylcholine; α3RRR, α3L210R; β4RRR, β4K208R/K210R; Bgtx, bungarotoxin; EB, epibatidine; ERAD, endoplasmic reticulum associated degradation; LGIC, ligand-gated ion channel; nAChR, nicotinic acetylcholine receptor; PCR, polymerase chain reaction; WT, wild type

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[2] Numbering corresponds to nAChR *Torpedo californica* mature α1 sequence and will be used hereafter for all subunits.

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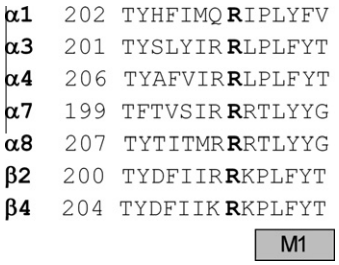

Fig. 1. Aligned amino acid sequences of the pre-M1 and adjacent regions of the *Torpedo californica* α1, bovine α7, α3 and β4, chick α8 and human α4 and β2 nAChR subunits. Numbering corresponds to mature sequences. The invariant R is shown in bold.

sary for the assembly of α3β4 receptors, whereas the same amino acid in the β4 subunit plays a minor role in the biogenesis of α3β4 receptors [7].

In all α and β neuronal nAChR subunits the residue at position 208 is positively charged, either R or K. In αBgtx-sensitive neuronal α nAChR subunits the residue at position 210 is R. In contrast, in αBgtx-insensitive neuronal α nAChR subunits the residue at position 210 is the aliphatic L (Fig. 1). To determine the role of residues flanking the R209 amino acid in the biogenesis and function of neuronal nAChRs, we performed mutations of residues 208 and 210 in homomeric α7 and heteromeric α3β4 nAChRs.

2. Materials and methods

2.1. Site directed mutagenesis and oocyte expression

Point mutations of bovine α7, α3 and β4 nAChR subunits cloned in pSP64T vector [8], in vitro synthesis of capped mRNA and injection of cRNAs into defoliculated *Xenopus laevi* oocytes was performed as described [7]. All experiments were performed within 3–6 days after cRNA injection.

2.2. [^{125}I] α-Bgtx and [^{3}H] epibatidine (EB) binding assays

Total and surface expression of [^{125}I]-α-Bgtx binding sites was done as described [7]. Microsomal fraction from *Xenopus* oocytes was obtained as described [10] with minor modifications. [^{3}H]-EB equilibrium binding to oocytes microsomal fraction was done as described [11]. Experimental data of saturation experiments were fitted to a one-binding site saturation model using GraphPad Prism 5 software. Surface expression of α3β4 nAChRs was measured as described [12].

2.3. Sucrose gradients

Sucrose gradient analysis of α-Bgtx sensitive AChRs was performed as described [7]. Solubilized oocytes expressing α7 subunits were labeled with [^{125}I]-α-Bgtx and layered onto 11 ml sucrose gradients (5–20%). After sedimentation of nAChRs, 250 μl-aliquots were collected and immunoprecipitated with mAb 319 [9]. Oocytes expressing α3β4 nAChRs were solubilized and sedimented as before. Collected aliquots were incubated with [^{3}H]-EB and mAb 35 and was radioactivity measured. Control gradients with purified *Torpedo* nAChR were run in parallel.

2.4. Metabolic labeling

Oocytes were labeled for 3 or 6 h at 18 °C in Barth's solution containing 0.5 mCi/ml of [^{35}S]-methionine. The labelling was stopped with the addition of Barth's solution containing 5 mM methionine. The oocytes that were labeled for 3 h were solubilized and immunoprecipitated as before, and radioactivity was measured. Oocytes that were chased (6 h labeling) were washed twice with Barth's solution containing 5 mM methionine and then incubated at 18 °C for 18 h.

3. Results

3.1. Expression and assembly of α7 subunit R208 mutants

cRNAs corresponding to α7R208 mutants were injected into *Xenopus* oocytes and the specific total and surface expression of [^{125}I]-α-Bgtx binding sites was measured. Total expression of α7R208K nAChRs was reduced by 50%. Total expression of α7R208E and α7R208T was decreased to an even larger degree. In the case of the α7R209L mutant no [^{125}I]-α-Bgtx binding sites were detected (Fig. 2A). For all mutants tested the extent of surface expression was consistent with the total number of [^{125}I]-α-Bgtx binding sites (Fig. 2A). Thus, the transport of assembled mutated receptors to the cell surface was preserved. Results were not due to a change in the affinity of [^{125}I]-α-Bgtx, as Kd's of [^{125}I]-α-Bgtx binding were similar for wild type (WT) and mutated receptors (Fig. 2B). The size of expressed receptors is consistent with pentameric oligomers, since sucrose gradients of both control and mutated receptors revealed complexes similar to Torpedo AChR monomers (Fig. 3). These results imply that a positively charged residue at position 208 is necessary for the assembly of mutant receptors. Negatively charged or hydroxylic residues are able to assemble, but less efficiently.

3.2. Expression and assembly of α7 subunit R210 mutants

No expression of α7R210E, α7R210T α7R210L mutants was evident, implying that either the synthesis, folding and assembly of mutant receptors was impaired or that the primary translation product was degraded. On the contrary, total binding of [^{125}I]-α-Bgtx to α7R210K receptors was similar to α7 WT (Fig. 2A). The Kd of [^{125}I]-α-Bgtx for α7R210K receptors was similar to α7WT nAChRs (Fig. 2B). Assembled α7R209K nAChRs were able to reach the oocyte surface to the same extent as α7WT nAChRs. The size of expressed receptors is consistent with pentameric oligomers, since sucrose gradients of both control and mutated receptors revealed complexes similar to Torpedo AChR monomers (Fig. 4). To further investigate if α7R210E, α7R210T and α7R210L mutants are assembled into pentamers but are not able to bind [^{125}I] α-Bgtx, oocytes were labeled with [^{35}S] methionine and nAChRs were immunoprecipitated with a specific monoclonal antibody (Fig. 2C). After 3 h of labeling, expression of mutants studied was significant albeit lower than α7WT. In contrast, expression of mutants chased for 18 h was practically absent. These results suggest that α7R210E, α7R210T and α7R210L subunits are synthesized, but not properly folded or assembled and therefore degraded. Altogether, results indicate that a positively charged residue at position 210 is essential for the correct assembly of α7 nAChRs.

3.3. Expression and assembly of α7 subunit R208/R210 double mutants

As in the case of α7 R210, but not α7 R208, only α7R208K/R210K was able to assemble. Assembled receptors were able to reach the oocyte surface, as the expression total and surface [^{125}I]-α-Bgtx binding sites was similar to α7 WT. Double-mutants α7R208E/R210E, α7R208T/R210T or α7R208L/R210L yielded no expression of nAChRs (Fig. 2A). The affinity of [^{125}I]-α-Bgtx for