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Role of loop 9 on the function of neuronal nicotinic receptors

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ABSTRACT

We have studied the role of loop 9 in the function of neuronal nicotinic receptors. By systematically mutating the residues in the loop we have determined that the most important amino acids determining the coupling of binding to gating are the ones closer to the transmembrane region. Single mutations at location E173 in homomeric $\alpha 7$ receptors destroyed their function by completely abolishing the current while preserving the expression at the membrane. In contrast, heteromeric receptor $\alpha 3\beta 4$ with the same mutations retained some function. We conclude that loop 9 has a different role in the function of homomeric and heteromeric receptors.

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1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChR) are ligand-gated ion channels composed of five homologous or identical subunits [5,19]. Structural information of nAChR have been obtained at atomic resolution from Torpedo receptors [26], *homologous* molluscan ACh-binding proteins [3], and from the extracellular domain of mouse $\alpha 1$ subunits [7]. In all cases the extracellular domain of each subunit has a similar structural organization containing an N-terminal α -helix, a core of 10 β -strands, and several loops linking the different elements. In Torpedo receptors, the first ones from which we have structural data on the transmembrane regions, loops 2, 7 and 9 are part of the interface with the transmembrane domain and have been implicated in coupling ligand binding to channel opening [25]. In particular, it has been hypothesized that agonist binding induces the movement of loop 9 (linking sheets $\beta 8$ and $\beta 9$) [21]. In addition, the X-ray structure of two complete prokaryotic ligand-gated ion channel analogs has shown similar features [2,14].

We had previously studied in detail the interactions of loops 2 and 7 with the M2–M3 linker that are involved in the gating of the $\alpha 7$ nicotinic receptor [1,23]. Loops 2, 3 and the M2–M3 linker form the interface between the extracellular and transmembrane receptor domains. In contrast with loops 2 and 7, loop 9 (L9) does not seem to be so close to the transmembrane domains in the structural models, and its role has not been studied exhaustively. However, its large size and presumably large flexibility make it a very good candidate for

interactions not only with the transmembrane domains, but also with extracellular regions of neighboring subunits.

On the other hand, studies done in muscle nAChRs have suggested that only in α subunits residues of loops 2 and 7 contribute appreciably to coupling binding to gating, whereas residues at equivalent positions of non- α -subunits show negligible coupling [18]. Thus, we decided to undertake a systematic study of the role of the residues in L9 in the gating of both the homomeric $\alpha 7$ and the heteromeric $\alpha 3\beta 4$ receptors where the role of residues in loop 9 could be studied in α and non- α subunits.

2. Materials and methods

2.1. Generation of mutants

The bovine $\alpha 7$ cDNA [11] was cloned in a derivative of the pSP64T vector [16] containing part of the pBluescript polylinker. Heteromeric nAChRs made of human $\alpha 3$ and rat $\beta 4$ subunits were also used. 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. Modified $\alpha 3$ [20] or $\beta 4$ [24] subunits able to bind α -Bgt (designated $\alpha 3^*$ and $\beta 4^*$ henceforth) were always used in combination with the corresponding non-modified subunit where the mutation was introduced.

2.2. Oocyte expression

Capped mRNA was synthesized *in vitro* using SP6 RNA polymerase, the mMESSAGING-mMACHINE kit (Applied Biosystems, Madrid, Spain) and the pSP64T derivative mentioned above. Defoliated *Xenopus*

Abbreviations: L9, loop 9; ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; α -Bgt, α -bungarotoxin; WT, wild-type

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leavis oocytes were injected with 5 ng of total cRNA in 50 nl of sterile water. All experiments were performed within 2–3 days after cRNA injection. mRNA of wild-type receptors was injected into oocytes from the same frog every time a mutant was tested. Consequently, mutant expression was expressed as a percentage of wild-type receptor expression observed in the same experiment.

2.3. [¹²⁵I]- α -bungarotoxin binding assays

Specific surface expression of [¹²⁵I]- α -bungarotoxin (α -Bgt) (PerkinElmer España, Madrid, Spain) binding sites in α 7 receptors was tested with 10 nM [¹²⁵I]- α -Bgt as described [10].

Briefly, 40 oocytes located in 24-well plates were pre-incubated for 15 min with Barth's buffer (in mM: 84 NaCl, 1 KCl, 2.4 HCO₃Na, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 7.5 Tris-Cl pH 7.4) containing 5% of fetal calf serum and further incubated in the same medium with 10 nM [¹²⁵I]- α -Bgt for 2 h at 18 °C in a final volume of 250 μ l. At the end of the incubation, unbound [¹²⁵I]- α -Bgt was removed, oocytes were passed to 6-well plates, washed five times with 4 ml Barth's buffer and bound radioactivity was counted.

Binding of [¹²⁵I]- α -Bgt to heteromeric α 3 β 4 receptors was performed in the same way, except for the use of a final concentration of 67 nM [¹²⁵I]- α -Bgt. In this case mutant α 3 [20] or β 4 [24] subunits able to bind α -Bgt were used in combination with the corresponding non-binding subunit.

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 in order to discount potential decreases in toxin affinity.

All binding experiments with mutant receptors (and the corresponding WT) were repeated with two or three different donors in successive weeks and the ratio of α -Bgt binding for mutant to WT was expressed as mean \pm s.e.m.

2.4. Electrophysiological recordings

Electrophysiological recordings were carried out as previously described [10]. Extracellular solution contained (in mM): NaCl 82.5, KCl 2.5, BaCl₂ 2.5, MgCl₂ 1 and HEPES 5 (pH 7.4). This solution, by substituting calcium by barium, diminishes the activation of calcium-activated chloride currents. The velocity of application of agonists was 18–22 ml min⁻¹. Functional expression of each construct was estimated as the peak ionic current evoked by 4 s application of 1 or 3 mM ACh at -80 mV and no correction for desensitization was made. All experiments were performed at room temperature (22 °C). Data analysis was performed with Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Dose–response curves were fitted using a nonlinear least squares algorithm to the Hill equation, $I/I_{\max} = 1 / (1 + (EC_{50}/C)^h)$. Data are expressed as mean \pm s.e.m. Statistical significance was calculated by one-way ANOVA test and, when a significant *F* value was obtained, by Bonferroni's multiple comparison test. The null hypothesis was rejected when $p < 0.05$.

As with the binding experiments, all electrophysiological recordings of the currents of the mutant receptors (and the corresponding WT) were repeated with two or three different donors in successive weeks. Each week at least seven cells expressing mutant and the same number of cells expressing WT were recorded. The ratio of peak currents for mutant to WT was expressed as mean \pm s.e.m.

3. Results

3.1. Alanine scanning mutagenesis of loop 9

Fig. 1 shows, at increasing level of detail, part of a structural homology model of the bovine α 7 subunit depicting in color the amino

acids of L9 explored in the present study. We started with an alanine scanning mutagenesis study of all these residues.

Plasma membrane expression of mutant nAChRs was monitored by measuring α -Bgt binding sites at the external surface of oocytes, and the functional state of these receptors was tested by recording the ionic currents evoked by a pulse of ACh 1 mM. As an internal control in all experiments, all current and binding data were compared to wild-type receptors, and expressed as a fraction of the corresponding values obtained in WT α 7 nAChRs. The results are shown in Fig. 2. We also calculated the value of C/B to compare the functionality of all mutants.

As seen in Fig. 2, most of the mutants had a significant reduction in expression, most notably, D164A, Y168A and N171A that had an expression less than 30% of the control value. On the other hand the value of the currents for these mutants was also reduced even in a large proportion, 21%, 12% and 20% respectively, thus giving C/B values between 0.5 and 0.8 indicating that those mutants had lost some of their ability to function normally.

In contrast, for mutant M160A, although there was a decrease in expression to $61 \pm 5\%$ of WT, the decrease in current was to only $70 \pm 20\%$ of WT, thus resulting in an insignificant increase in function ($114 \pm 23\%$). This effect was most pronounced in mutant I169A where a decrease to $43 \pm 4\%$ in binding was accompanied by only a decrease to $62 \pm 6\%$ in the currents, thus giving an increase in function to $145 \pm 9\%$ of control. In a different way, mutant G167A also showed a small increase in function similar to M160A, but in this case without a significant reduction in expression.

Residues close to the end of L9 turned out to be essential for the function of the receptor; this is shown in mutant G172A that with only a small reduction in expression, $72 \pm 2\%$ of WT, had a dramatic decrease in current: only $4 \pm 4\%$ of control. The next residue in the sequence, E173 when mutated to alanine gave a receptor with a moderate expression, $47 \pm 14\%$ of WT, but with undetectable currents. Interestingly, the alanine mutation in first residue after L9, the highly conserved W174 produced receptors that were not expressed in the membrane (data not shown). In the rest of the study we concentrated in the last two positions in L9 to further investigate its role in the function of the receptor.

Fig. 2B shows a summary of these results in pictorial form by applying a linear color code scale on the current–binding ratio (C/B) to the side chains of the residues of L9.

3.2. Role of the end of L9 on the gating of the homomeric receptor α 7

After identifying that alanine mutants at the end of L9 strongly affect the function of the receptors, and considering that alanine residues have a hydrophobic side chain, we also wanted to study the effect of other mutations with amino acids of different characteristics, i.e. with polar (Ser) or charged side chains of different sign (Asp, and Lys). Mutant G172S gave, consistently, values of expression close to the control and currents about ten times smaller but still measurable so we used this mutant to pursue the study. The resulting pharmacology of this mutated receptor can be seen in Fig. 3.

The average values for ACh concentration–response curves parameters (EC_{50} , nM) were for α 7 ($47 \pm 3 \mu$ M, 1.87 ± 0.09) and for G172S ($147 \pm 4 \mu$ M, 1.49 ± 0.04), whereas for DMPP were: α 7 ($10 \pm 1 \mu$ M, 3.03 ± 0.15) and G172S ($30 \pm 4 \mu$ M, 1.97 ± 0.30), $n = 4$ in all cases. In α 7 DMPP gave maximal currents very close to those elicited by ACh, their ratio being 0.97 ± 0.01 whereas in G172S this ratio was only 0.66 ± 0.04 (data not shown). Thus G172S has a concentration–response curve shifted to the right by a factor of 3 both for ACh and DMPP, and with maximal currents that are approximately ten times smaller.

The kinetics of the currents was also affected by the mutation. At saturating concentrations, the rising time from 10% to 90% of the peak was 21 ± 4 ms ($n = 6$) for α 7 and 13 ± 1 ($n = 10$) ms for G172S, and the

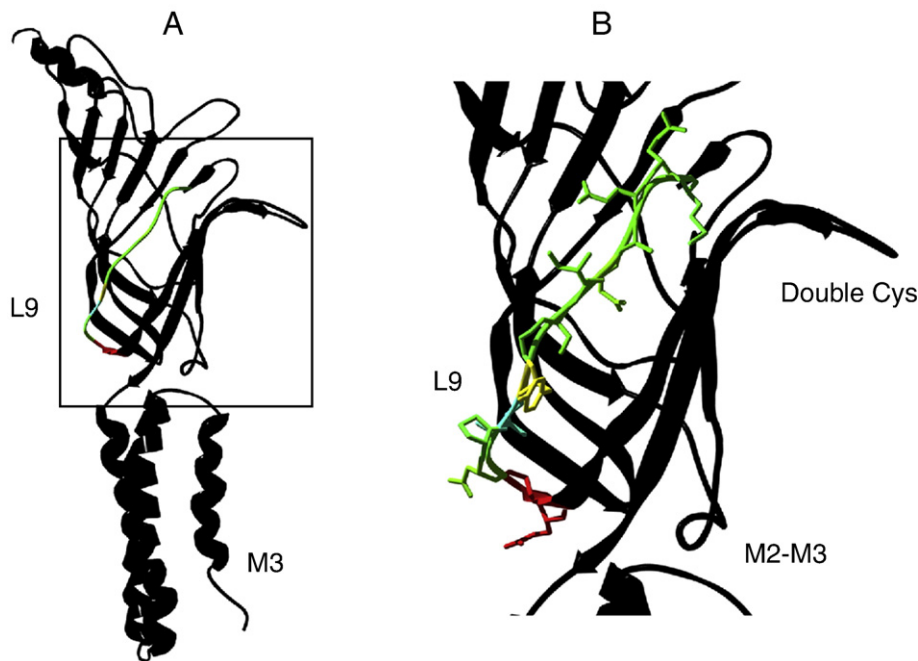


Fig. 1. Location of the amino acids in L9 examined in this study. Data are from the bovine $\alpha 7$ subunit homology model based on the electron microscopic studies of Unwin [26] (PDB code: 2bg9). A. Overview of the structure of a single subunit where L9 appears in color. B. Zoom of the region selected in A showing in color the side chains of residues in L9.

decaying time from the peak to 50% was 99 ± 9 ms for $\alpha 7$ and 42 ± 4 ms for G172S. Thus both processes were approximately two times faster in the mutant.

When a charge is introduced at position G172 the receptor is still expressed in the membrane, however the current is strongly reduced in the case of the negatively charged mutant G172D, and undetectable in the case of the positively charged one, mutant G172K, that abolishes the current completely, Fig. 4.

In contrast, mutations in the next position, E173, produced receptors with a slight reduction in the expression but complete elimination of the current in all cases, regardless if the mutation neutralizes, reverses or preserves the charge, as in mutations E173Q, E173K and E173D respectively, Fig. 4. No current was detected either

when the concentration of ACh applied was increased from 1 mM to 10 mM.

3.3. Role of the end of L9 on the gating of the heteromeric receptor $\alpha 3\beta 4$

The previously observed reduction in C/B suggests that residues G172 and E173 play an important role in the gating of the homomeric $\alpha 7$ receptor. We wondered whether the same happened in heteromers and if so, whether a difference would be observed depending on which subunit, α or β , would carry the mutations. The results of these experiments are shown in Fig. 4 for the slowly-desensitizing receptor $\alpha 3\beta 4$. Contrarily to what happened with mutations in $\alpha 7$ the corresponding mutations in $\alpha 3$ had a smaller reduction in C/B

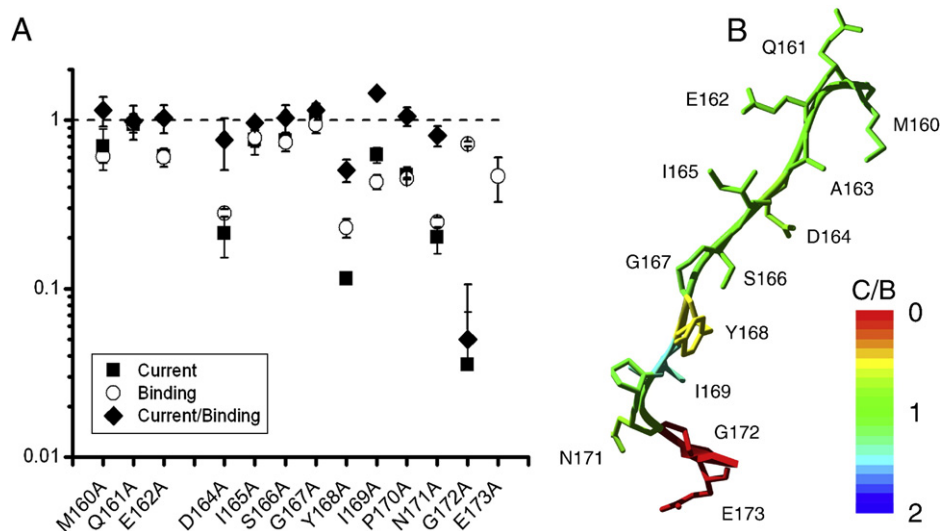


Fig. 2. Current, binding and current/binding ratio for alanine mutants in $\alpha 7$ receptors. A. Normalized values of current (squares), binding (circles) and current/binding ratio (diamonds) for all residues of L9 mutated to alanine. All data have been normalized to the corresponding values obtained in the WT $\alpha 7$ receptor. B. Detail of residues in L9 labeled and colored in a scale representing the effect on current/binding ratio (C/B) when the residue is mutated to alanine.

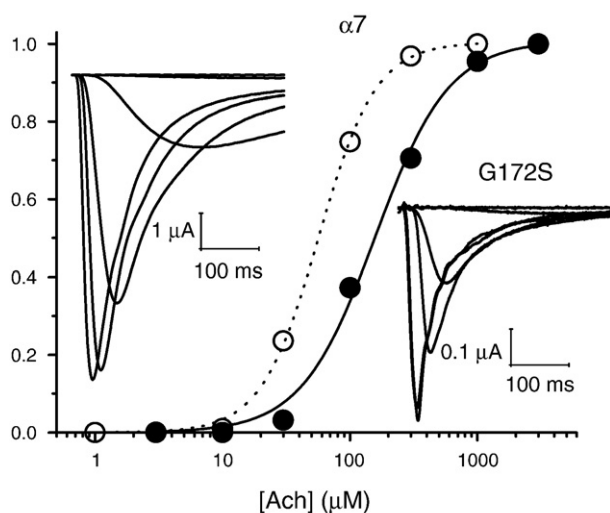


Fig. 3. Concentration–response curves for WT and G127S. Normalized ACh concentration–current curves for two representative cells expressing $\alpha 7$ and G127S. Lines through data points are fits to the Hill equation. The data were obtained from the current records shown beside each curve. Note the 10 fold difference in the current scale.

both positions; the difference is especially notable for the glutamate mutants that produced receptors with small but measurable currents. This contrast is even more striking for the mutants in $\beta 4$ because the mutation had either no effect at all in C/B (mutant G174A), or only a relatively small effect (mutants at position E175).

When both subunits, $\alpha 3$ and $\beta 4$, were mutated simultaneously at location G174, the reduction of the maximum current was consistently larger than when only one subunit was mutated, but still smaller than the observed in homomeric $\alpha 7$ receptors. Finally, when the mutations were simultaneously introduced at location E175, they did not produce a further decrease of the current as compared with the one produced by the single mutation of $\alpha 3$ subunit, compare stars and squares in Fig. 4. Therefore, even in the case of receptors

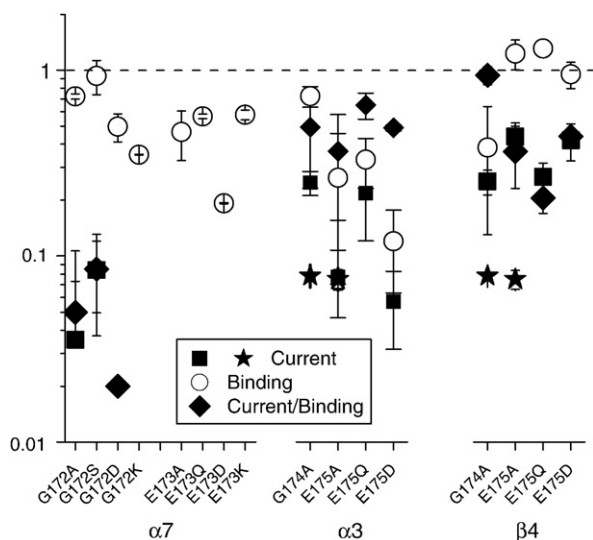


Fig. 4. Current, binding and current/binding ratio for mutants at the end of L9. Normalized values of current (squares, stars), binding (circles) and current/binding ratio (diamonds) for mutants of the last two residues of L9 in $\alpha 7$ and in subunits $\alpha 3$ or $\beta 4$ of $\alpha 3\beta 4$ receptors. Stars refer to current values obtained in heteromeric $\alpha 3\beta 4$ receptors whose five subunits have been mutated. In this case no α -Bgt binding could be determined. All data have been normalized to the values obtained in the WT $\alpha 7$ or $\alpha 3\beta 4$ receptors, or the corresponding modified receptors $\alpha 3\beta 4^*$ and $\alpha 3^*\beta 4$.

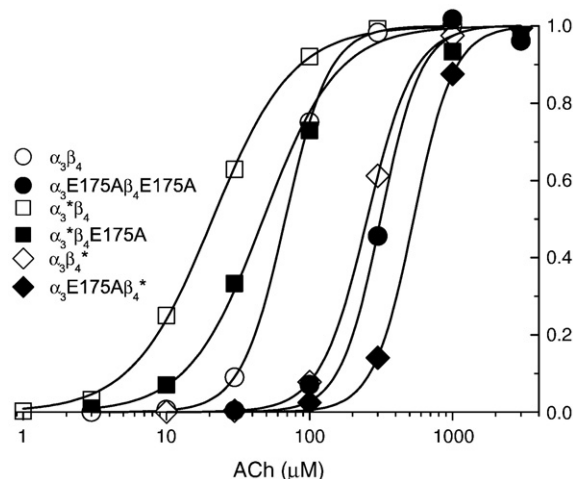


Fig. 5. Normalized ACh concentration–response curves of heteromeric nAChRs. Shown are wild-type $\alpha 3\beta 4$ and its mutant $\alpha 3E175A\beta 4E175A$, $\alpha 3^*\beta 4$ and its mutant $\alpha 3^*\beta 4E175A$, and $\alpha 3\beta 4^*$ and its mutant $\alpha 3E175A\beta 4^*$. Lines are fits to the Hill equation.

composed of five mutated subunits, the effects of mutating G174 and E175 were less pronounced in $\alpha 3\beta 4$ heteromeric receptors.

The possibility that mutations of E175 in either $\alpha 3$ or $\beta 4$ could be affecting the binding of ACh was investigated by analyzing concentration–response curves like the ones shown in Fig. 5. The modifications introduced into $\alpha 3$ or $\beta 4$ subunits to make them able to bind α -Bgt produced by themselves clear and opposite effects on the EC_{50} for ACh. However, when mutations at location E175 were introduced, consistent results were observed in both receptors, i.e. a shift in the curve towards higher concentrations. The average values of the fitted parameters for EC_{50} , and Hill coefficients are given in Table 1. The mutations produced statistically significant differences in EC_{50} ($p < 0.01$), but not in the Hill coefficient. On the other hand, the mutations did not produce a significant change in the kinetics of the currents (data not shown).

The magnitude of the shift in the concentration–response curves produced by mutations in $\alpha 3$ or $\beta 4$ subunits is small, approximately a factor of three as it was observed previously in $\alpha 7$ receptors. The small magnitude and direction of this effect is predicted as a consequence of the decrease in gating produced by the mutation. When mutations are present in both, $\alpha 3$ and $\beta 4$ subunits, within the same receptor, the change in EC_{50} is larger, approximately a factor of five, however, in this case we cannot assess whether this could be a consequence of decreased gating because we do not have expression data for these double mutant receptors.

4. Discussion

In this report we have investigated the role of L9 (also referred to as loop F) in the function of homomeric $\alpha 7$ and heteromeric $\alpha 3\beta 4$ neuronal nAChRs. The systematic mutation of all residues of L9 to alanine reveals that the end of L9 is especially important in the function of $\alpha 7$ receptors, as single mutations at the last residue in L9,

Table 1

Average values of fitted parameters (EC_{50} , and Hill coefficient) for concentration–response curves of ACh on heteromeric receptors.

Receptor	EC_{50} (μM)	nHill	N
$\alpha 3\beta 4$	61 ± 10	2.4 ± 0.4	4
$\alpha 3^*\beta 4$	18 ± 3	1.7 ± 0.3	6
$\alpha 3^*\beta 4E175A$	63 ± 7	1.4 ± 0.1	6
$\alpha 3\beta 4^*$	259 ± 13	3.2 ± 0.4	6
$\alpha 3E175A\beta 4^*$	559 ± 22	2.9 ± 0.1	5
$\alpha 3E175A\beta 4E175A$	321 ± 26	2.9 ± 0.6	4

E173, can abolish completely the function of the channel without affecting its expression in the membrane (Figs. 1 and 2). Interestingly, even the charge conserving mutation E173D formed non-functional receptors. This region of L9 sits at the interface between subunits so that it may interact with loops 5 and 10 that contribute to the ligand-binding site of the opposite subunit [26].

Mutations at the location previous to E173 in L9 (G172) produced receptors with small but measurable currents that allowed us to investigate the role of this part of L9 in coupling of binding to gating. Besides having a C/B about ten times smaller, the currents of mutant G172S were also about two fold faster both in the rising and decaying phase (Fig. 3). Both effects could contribute to the observed shift in the ACh concentration–response in the mutant without any change in the binding properties of the receptor [4]. On the other hand the mutation seemed to change the agonist specificity of the receptor because the ratio of peak currents for ACh and DMPP changed from 1 in WT to 0.6 in the mutant.

The special role of the conserved glutamate in L9 in modulating the activation of nAChRs has also been studied previously in homomeric non-desensitizing chick L247T $\alpha 7$ receptors by the substituted-cysteine accessibility method, concluding that receptor activation includes subunit rotation and/or intrasubunit conformational changes that move the corresponding residue E172 to a position away from the vestibule of the receptor [21]. In these modified receptors, the mutation E172C produced a decrease of the maximal current of 3.8-fold but a two orders of magnitude increase in the EC_{50} for ACh. We have ruled out the occurrence of a similar shift in EC_{50} in our mutated bovine $\alpha 7$ receptors because no current was detected when pulses of 10 mM ACh were applied (a concentration 200-fold greater than the EC_{50} for WT bovine $\alpha 7$ receptors). Thus, E173 seems to have an important role in the gating of homomeric WT bovine $\alpha 7$ receptors and not so much in modified receptors like mutant L247T $\alpha 7$ that already have an important alteration in their gating behavior.

The dramatic influence of E173 on the gating of $\alpha 7$ may be due to the homomeric nature of this receptor in contrast to the more modest effect on heteromeric receptors, Fig. 4. By using heteromeric $\alpha 3\beta 4$ receptors, and assuming a 2:3 stoichiometry [13], we were able to analyze the effect of mutating only two ($\alpha 3$) or three ($\beta 4$) subunits at a time. (Although there is no direct evidence of a fixed subunit stoichiometry for $\alpha 3\beta 4$ receptors expressed in oocytes, this is suggested by monophasic ACh concentration–response curves). In this case the mutant receptor was still functional although with a reduction in its ability to couple binding to gating, as measured by the C/B ratio, Fig. 4. In order to measure the number of heteromeric receptors in the membrane we used modified $\alpha 3$ or $\beta 4$ subunits in which the sequence of the $\alpha 1$ subunit C-loop is inserted [20,24]. Both modifications probably altered the binding properties of the receptors because the ACh concentration–response curves were shifted in opposite directions by three fold, Fig. 5, without a change in the magnitude of the current. Nevertheless, the mutation in the corresponding glutamate in $\alpha 3$ or $\beta 4$ subunits shifted the EC_{50} values by a factor close to 3, but in this case with a corresponding reduction in C/B. In these slowly-desensitizing receptors this shift in EC_{50} could be explained just by the reduction in C/B, [4]. Glutamate at the end of L9 has been shown to be a determinant of agonist affinity in muscle nAChRs [6,22], however the effect of E173 on ACh binding on neuronal nAChRs does not seem to be as important.

L9 has also been involved in the calcium binding site that regulates the potentiation of the receptor. Mutations in E172 in the $\alpha 7$ -V201-5HT₃ chimera have been shown to abolish calcium effects on ionic current amplitudes and agonist affinity [9]. Based on molecular models of chick $\alpha 7$ receptors, the same glutamate residue has been proposed to form a Ca²⁺-binding site pocket with aspartate and glutamate residues of the neighboring subunit [17]. In chick L247T $\alpha 7$ receptors it has been proposed that E172 lines the extracellular vestibule being part of Ca²⁺ binding sites that are important in both

ion permeation and modulation in those mutated receptors [8]. We could not pursue the study of the role of E173 on the calcium regulation of the bovine $\alpha 7$ receptor because all the mutants tested, including the mutation to glutamine used in previous studies, were non-functional.

The role of the glutamate in L9 in the modulatory effect of calcium could be studied in heteromeric receptors where, contrarily to what happens in $\alpha 7$, the mutation from glutamate to alanine produces functional receptors. This modulatory effect of calcium has also been previously observed in muscle and neuronal heteromeric receptors $\alpha 3\beta 4$ [27]. However, in our hands, the effect is seen only at low concentrations of ACh, 10 μ M or less, where the currents are too small to obtain reliable data, especially with the double mutant $\alpha 3E175A\beta 4E175A$. Interestingly, this heteromeric receptor, in which the five subunits contain the mutation E175A, consistently produced small (about ten times smaller than the control) but still measurable currents, as opposite to the similar mutant in $\alpha 7$ receptors, that did not produce currents at all. Thus, we can conclude that the requirement for E175 at the end of L9 is different in homomeric and heteromeric receptors.

Interactions between anionic residues in loops 9 and 10 of neighboring subunits in muscle have been investigated by a combination of natural and unnatural mutagenesis and a study of the change in the values of EC_{50} , but without measuring receptor expression in the membrane [12]. More recent studies however, suggest that there is no correlation between the rearrangements in loop 9 and channel opening in GABA receptors and that it is a component of the structural machinery that locks ligand into the agonist-binding site [15].

We consider that, in order to further understand neuronal nAChRs function, it would be interesting to study in more detail the possible interactions of the residues in L9 characterized here and the corresponding ones in the neighboring subunits by combining techniques that allow the measurement of receptor expression and function.

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