Charged Amino Acids of the N-terminal Domain Are Involved in Coupling Binding and Gating in α7 Nicotinic Receptors*

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Francisco Sala, José Mulet, Salvador Sala, Susana Gerber, and Manuel Criado‡

From the Instituto de Neurociencias, Universidad Miguel Hernández-Consejo Superior de Investigaciones Científicas, Apartado 18, 03550-Sant Joan d’Alacant, Alicante, Spain

Binding of agonists to nicotinic acetylcholine receptors generates a sequence of conformational changes resulting in channel opening. Previously, we have shown that the aspartate residue Asp-266 at the M2-M3 linker of the α7 nicotinic receptor is involved in connecting binding and gating. High resolution structural data suggest that this region could interact with the so-called loops 2 and 7 of the extracellular N-terminal region. In this case, certain charged amino acids present in these loops could integrate together with Asp-266 and other amino acids, a mechanism involved in channel activation. To test this hypothesis, all charged residues in these loops, Asp-42, Asp-44, Glu-45, Lys-46, Asp-128, Arg-130, and Asp-135, were substituted with other amino acids, and expression levels and electrophysiological responses of mutant receptors were determined. Mutants at positions Glu-45, Lys-46, and Asp-135 exhibited poor or null functional responses to different nicotinic agonists regardless of significant membrane expression, whereas D128A showed a gain of function effect. Because the double reverse charge mutant K46D/D266K did not restore receptor function, a gating mechanism controlled by the pairwise electrostatic interaction between these residues is not likely. Rather, a network of interactions formed by residues Lys-46, Asp-128, Asp-135, Asp-266, and possibly others appears to link agonist binding to channel gating.

The nicotinic acetylcholine receptor (nAChR) is a member of the superfamily of ligand-gated ion channels that mediates fast synaptic transmission in nerve and muscle cells (1, 2). Agonist binding to the nAChR triggers a signal that must be transmitted to the channel gate probably through conformational changes (3). Charged amino acids located in the extracellular M2-M3 linker have been reported to affect coupling in glycinergic (GlyR) (4, 5), γ-aminobutyric acid (GABAAR) (6, 7), and nicotinic receptors (nAChRs) (8, 9). Electron micrograph (10) and crystallographical (11) studies have suggested that the M2-M3 linker could interact with residues at the so-called loops 2 and 7 of the extracellular domain, and it has been postulated that this interaction could be involved in the mechanism of receptor activation (10). Recently, Kash et al. (12) have demonstrated the relevance for channel gating of a direct electrostatic interaction between the positively charged Lys-279 residue in the M2-M3 linker of the GABAAR and negatively charged residues of loops 2 and 7. Electrostatic interactions also appear to be relevant for gating of GlyRs (13, 14) apparently in a different way than in the case of the GABAAR. We have shown previously that the negatively charged Asp-266 residue in the M2-M3 linker of α7 nAChRs (8) and an equivalent residue in other neuronal nAChRs (15) are involved in coupling agonist binding and gating; however, no studies have been reported on a similar role of charged residues in loops 2 and 7 of nAChRs. Identification of such residues would be of relevance because it can provide insights into the gating mechanism of nAChRs and allow comparisons with data obtained from other ligand-gated ion channels (12–14). We report here that mutations of amino acids Glu-45 and Lys-46 (loop 2) and Asp-135 (loop 7) of the α7 subunit caused dramatic reductions in the currents evoked by different agonists, whereas some mutants at Asp-128 showed the opposite effect suggesting that these residues play an important role in the transduction of agonist binding into channel activation.

MATERIALS AND METHODS

Generation of Mutants of the Bovine α7 Subunit—The bovine α7 cDNA (16) was cloned in a derivative of the pSP64T vector (17) containing part of the pBluescript polylinker. The appropriate DNA cassettes were generated by PCR (25 cycles at 94 °C for 10 s, 60 °C for 30 s, 72 °C for 45 s) and used to substitute original segments of the α7 subunit through restriction enzyme digestions. For this purpose we used restriction enzyme sites present in the original cDNA sequence such as a PstI site, corresponding to amino acids Leu-38 and Gln-39 just at the N-terminal region of loop 2, a HinFII site at its C terminus at positions Leu-50 and Thr-51, a SacI site corresponding to amino acids Ser-123 and Ser-124 at the N-terminal region of loop 7, and an Styl site corresponding to amino acids Ser-152 and Leu-153. In addition, silent mutations were introduced to generate two restriction enzyme sites useful for mutants construction, an AatII site corresponding to amino acids Gly-144 and Ser-145. To generate the mutants we annealed single-stranded oligonucleotides with the desired sequences and proper single-strand ends that could be easily ligated to the ends generated by the restriction enzymes mentioned above.

Oocyte Expression—Capped mRNA was synthesized in vitro using SP6 RNA polymerase, the mMESSAGEMACHINE kit (Ambion) and the pSP64T derivative mentioned above. Defolliculated Xenopus laevis oocytes were injected with 5 ng of total cRNA in 50 nl of sterile water. All experiments were performed within 3–4 days after cRNA injection. Wild-type α7 mRNA 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 α7 expression observed in the same experiment.

125I-a-Bungarotoxin (α-Bgt) Binding Assays—Specific surface expression of 125I-a-Bgt binding sites was tested with 5 nM 125I-a-Bgt as
Electrophysiological Recordings—Electrophysiological recordings were done as described previously (18). Briefly, oocytes were incubated with 5 nM $^{125}$I-α-Bgt for 2 h at 18 °C. At the end of the incubation, unbound $^{125}$I-α-Bgt was removed, oocytes were washed, and bound radioactivity was counted. Nonspecific binding was determined using noninoculated oocytes.

Expression of nAChRs was monitored by measuring dose-response curves by measuring the peak ionic current evoked by 1-s application of 1 or 3 mM ACh. It is important to note that the half-maximal response, $h$, is the agonist concentration.

RESULTS

A Charged to Alanine Scanning Mutagenesis Study of Loops 2 and 7—As an initial approach to determine which of the charged residues present in loops 2 and 7 of the nAChR α7 subunit play a role in the mechanism of channel activation (see Fig. 1), an Ala-scanning mutagenesis study was carried out. Expression of nAChRs was monitored by measuring α-Bgt binding sites at the external surface of oocytes, whereas the functional state of these receptors was tested by recording the ionic currents evoked by the wild-type receptors with similar decrease in functional responses. Therefore, it can be concluded that residues Glu-45, Lys-46, Asp-128, and Asp-135 might be involved in the mechanism of channel activation and deserve further study.

No Amino Acid Can Substitute Glu-45 and Asp-135 without Impairing Channel Function—Replacement of Glu-45 by a positively charged lysine strongly reduced receptor expression (Fig. 3). By contrast, the more conservative mutants E45Q and E45D expressed a significant amount of nAChRs at the oocyte membrane (Fig. 3). However, as it was observed with mutant E45A, ACh did not evoke detectable currents. Although the lack of functional responses impaired more detailed studies of the mentioned mutants, these results suggest that the presence of a glutamate at position 45 is necessary for an adequate functional response.

Something similar took place at position 135. Thus, expression was strongly reduced to about 11% of the control in mutant D135A (Fig. 2), and the same happened with the more conservative mutations D135N and D135E (data not shown). The amount of mutant nAChRs present at the oocyte membrane was small, about 1.5 fmol of bound α-Bgt per oocyte, but not for gating. Mutant D44A barely produced nAChRs at the oocyte membrane, and no current was detected; therefore no conclusion about its involvement on channel activation could be drawn. Mutants E45A, K46A, and D135A showed reduced or no functional responses that did not match the amount of nAChRs present at the oocyte membrane. By contrast, mutant D128A showed a relatively high functional response not correlated with the strong decrease observed in nAChR expression. Finally, mutant R130A showed a moderate decrease in receptor expression with similar decrease in functional responses. Therefore, it can be concluded that residues Glu-45, Lys-46, Asp-128, and Asp-135 might be involved in the mechanism of channel activation and deserve further study.

A Charged to Alanine Scanning Mutagenesis Study of loops 2 and 7 of bovine α7 nAChRs. Expression of the indicated single mutants was tested by α-Bgt binding (black bars) and by the extent of the ionic currents evoked by application of 1 or 3 mM ACh (open bars). All data were normalized to those obtained with the wild-type α7 subunit, and means ± S.E. of at least two experiments from different donors (about 30 oocytes/experiment) are shown. Typical values obtained with the bovine α7 subunit were ~10 fmol of bound α-Bgt/oocyte and ~7 μA/oocyte at −80 mV.

Receptor expression strongly decreased when residue Asp-42 was mutated to Ala. Currents evoked by ACh in this mutant also decreased in a parallel way. Therefore, it seems that this negatively charged residue is important for nAChR expression but not for gating. Mutant D44A barely produced nAChRs at the oocyte membrane, and no current was detected; therefore no conclusion about its involvement on channel activation could be drawn. Mutants E45A, K46A, and D135A showed reduced or no functional responses that did not match the amount of nAChRs present at the oocyte membrane. By contrast, mutant D128A showed a relatively high functional response not correlated with the strong decrease observed in nAChR expression. Finally, mutant R130A showed a moderate decrease in receptor expression with similar decrease in functional responses. Therefore, it can be concluded that residues Glu-45, Lys-46, Asp-128, and Asp-135 might be involved in the mechanism of channel activation and deserve further study.

FIG. 2. Effect of alanine mutations in the charged residues of loop 2 and 7 of bovine α7 nAChRs. Expression of the indicated single mutants was tested by α-Bgt binding (black bars) and by the extent of the ionic currents evoked by application of 1 or 3 mM ACh (open bars). All data were normalized to those obtained with the wild-type α7 subunit, and means ± S.E. of at least two experiments from different donors (about 30 oocytes/experiment) are shown. Typical values obtained with the bovine α7 subunit were ~10 fmol of bound α-Bgt/oocyte and ~7 μA/oocyte at −80 mV.

FIG. 1. A schematic representation of loops 2 and 7 and the M2-M3 linker of α7 nAChRs. This schematic, based on the crystallographic data of Brejc et al. (11) and the electron microscopy studies of Miyazawa et al. (10), depicts amino acids by circles, the black ones being the charged residues studied in this work.
A Positively Charged Residue Is Needed at Position 46 for Efficient Channel Gating—Mutant K46A reached expression levels similar to the wild-type receptor; however currents evoked by ACh were significantly lower, suggesting that gating was impaired (see below for a discussion on desensitization). This effect was even stronger with mutant K46E (Fig. 4), which was unable to show functional responses despite expressing a significant amount of receptors at the oocyte membrane (about half of the control). The same happened with mutant K46C (Fig. 4), although in this case expression levels decreased strongly. By contrast, substitution of lysine by the other positively charged amino acid, arginine (Fig. 4), barely affected expression and current levels, suggesting the requirement of a positive charge at position 46 for correct gating.

As mutant K46A showed reduced but still well defined ionic currents, dose-response relationships could be obtained for five different agonists (Fig. 5 and Table I). In wild-type receptors, dimethylphenylpiperazinium, nicotine, cytisine, and choline evoked maximal responses ranging 80–100% of the control (ACh). However, in mutant K46A all responses to these four agonists were also reduced with respect to ACh (down to 60%), suggesting again a modification in the gating mechanism. Moreover, upon continuous stimulation of agonist, peak currents decayed at the same rate in all receptors and with several agonists (data not shown), arguing against effects on gross desensitization. In all cases changes in EC₅₀ were not large, between 3- and 7-fold higher in mutant receptors, therefore, less than one order of magnitude. Thus, the observed changes could be mostly caused by alterations of the gating characteristics rather than to large modifications of agonist binding or desensitization properties.

Is There a Salt Bridge between Lys-46 and Asp-266?—As previously indicated, Kash et al. (12) have demonstrated the relevance for channel gating of a direct electrostatic interaction between a positively charged residue in the M2-M3 linker of the GABAAR and some negatively charged residues of loops 2 and 7. One example of evidence for that was that the double reverse charge mutation restores receptor function that was previously lost with the single mutants. For this reason we also tested the double mutant K46D/D266K and the corresponding single mutants K46D and D266K in the α7 subunit (Fig. 6).

Both single mutants yielded a significant amount of receptors at the oocyte membrane, 25% of the observed with wild-type α7 subunits, but these receptors were not activated by ACh (Fig.

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**FIG. 3.** Effect of several mutations of Glu-45 on functional expression of α7 nAChRs. The indicated single mutants were tested as in the legend of Fig. 2.

**FIG. 4.** Effect of several mutations of Lys-46 on functional expression of α7 nAChRs. The indicated single mutants were tested as in the legend of Fig. 2.

**FIG. 5.** A comparison between wild-type α7 nAChRs and K46A mutants. A, inward currents obtained upon 1-s stimulation with ACh (horizontal bars) of wild-type (wt) α7 nAChRs and K46A mutants. Traces represent currents obtained at −80 mV with the ACh concentrations indicated on top. B, dose-response curves of oocytes expressing wild-type α7 (filled squares) nAChRs and K46A mutants (open circles) for ACh (left) and dimethylphenylpiperazinium (right). Data points represent means ± S.E. obtained in 7–9 oocytes from three donors. Continuous lines represent fits of data to the Hill equation (see Table I for relative Iₘₐₓ, EC₅₀, and Hill coefficient values).
FIG. 6. Effect of the double reverse charge mutant K46D/D266K on functional properties of a7 nAChRs. A, the expression of single mutants K46D and D266K and the double mutant K46D/D266K is shown in the same conditions than in Fig. 2. B, co-injection of wild-type (wt) and single and double mutants from A. The expression of wild-type a7 nAChRs alone or co-injected 1:4 with the indicated mutants is shown in the same conditions as in Fig. 2. In A and B, numbers in parentheses beside the bars represent the normalized maximal currents divided by the value obtained in the binding experiments for the same combination. C, dose-response curves of oocytes expressing wild-type a7 (filled squares) nAChRs alone or co-injected 1:4 with K46D/D266K mutants (open circles) for ACh (left) and choline (Cho, right). Data points are normalized to the current obtained with 1 mM ACh and represent the mean ± S.E. obtained in 3–5 oocytes from two donors. Continuous lines represent fits of data to the Hill equation. EC_{50} and Hill coefficients were, respectively, 37 ± 2 μM and 1.33 (wild-type, ACh); 100 ± 3 μM and 1.07 (wild-type + K46D/D266K, ACh); 235 ± 12 μM and 1.96 (wild-type, choline); 793 ± 38 μM and 1.14 (wild-type + K46D/D266K, choline).

6A). In the case of the double mutant K46D/D266K, no nAChRs were detected at the oocyte membrane suggesting that the double mutation is deleterious for receptor expression (Fig. 6A).

An alternative strategy was then carried out, consisting of co-injecting wild-type and double mutant subunit RNAs in a proportion of 1 to 4, expecting to get expression of receptors in which mutant subunits would have been incorporated. These receptors could be compared then with the ones produced by only wild-type a7 RNA and also by co-injection of single mutants K46D and D266K with wild-type a7 in the same conditions. As shown in Fig. 6B, a significant amount of receptors was present at the oocyte membrane in all cases. Co-injection of a7 with either the single mutant K46D or the double mutant K46D/D266K produced ~30% of the receptors observed with wild-type a7, whereas expression was lower with D266K. When the single mutant K46D was present, these receptors were not functional, whereas the ones with D266K and specially the double mutant showed significant currents, although to a lower degree (36 and 44% of wild-type a7 nAChRs alone) than expected from the extent of expressed receptors. The possibility that these receptors are only made by a7 subunits because the double mutant subunits were unable to assemble nAChRs appears unlikely, as in this case the currents evoked by ACh should be similar to the ones with a7 subunits alone; therefore, the currents would be larger if we consider the level of receptor expression. Moreover, the receptors obtained with the co-injection of the double mutant showed dose-response relationships different from the ones obtained with wild-type subunits (Fig. 6C), with larger EC_{50} for ACh and choline. In fact, these data confirm that although the double reverse charge mutation allows the expression of functional receptors when co-injected with wild-type a7 subunits, it cannot restore the normal functionality observed when a7 subunits are alone.

Mutants at Position 128 Show Heterogeneous Behavior—Mutant D128A reached low expression levels, ~15% of the observed with wild-type receptors. However, currents evoked by ACh were only reduced to about a half (Fig. 2) of the control, suggesting a certain “gain of function” effect. The possibility that this mutant had reduced affinity for α-Bgt (therefore showing reduced expression) was discarded because a higher concentration of α-Bgt labeled the same amount of nAChRs. The conservative mutant D128E showed a similar behavior (Fig. 7), with current levels close to the a7 controls despite that expression decreased to less than half of the controls. Other mutants like D128K and D128N did not yield any receptor expression (Fig. 7).

Mutant D128A was studied in more detail by obtaining dose-response relationships for five different agonists (Table I). With the exception of choline, the relative increase in maximal responses is smaller for all other agonists when compared with...
ACh, suggesting again that the gating mechanism has been altered. Once again changes in desensitization rates were not apparent (not shown). With this gain of function effect increases in agonist potency should be expected, but the EC\textsubscript{50} values for nicotine and ACh were the same as the ones obtained for a\textsubscript{7} wild-type receptors, whereas changes in the EC\textsubscript{50} values for the other agonists were small, between 1.7- and 2.8-fold higher in mutant receptors. Though a neat distinction between gating and binding could not be done when measuring macroscopic currents, the data suggest that the effect of the mutation is mainly on gating properties and is very small, if there is any effect at all, on binding properties.

**DISCUSSION**

The model of activation of the nAChR proposed by Unwin and co-workers (10, 20) highlights the participation of the M2-M3 linker on the gating process as "receiver" (21) for conformational changes initiated at the agonist binding site. Because these two regions do not seem to be in direct contact, an intermediate "actuator" (21) appears to be required. According to crystallographical data from the ACh binding protein (11), loops 2 and 7 are the best candidates for this role given their proximity to both the M2-M3 linker and the binding site. An excellent study combined mutagenesis of charged residues at the mentioned regions of the GABA\textsubscript{R} with electrophysiological and cysteine cross-linking experiments to show that a direct electrostatic interaction between a positively charged residue at the M2-M3 linker and negatively charged residues of loops 2 and 7 was required for channel gating (12). Such a direct interaction could not be demonstrated for the GlyR (13, 14), although several charged residues in loops 2 and 7 were implicated in channel gating. In the present study we tested two hypotheses: first, that gating of a\textsubscript{7} nAChRs requires, like GABA\textsubscript{R} receptors, a direct electrostatic interaction between Asp-266 at the M2-M3 linker (8) and a positively charged residue at loop 2, Lys-46; second, that a broader network of charged residues present in these loops participates in the activation mechanism as it happens with the GlyR. For this reason, in addition to the positively charged residues mentioned above, negatively charged residues like Asp-42, Asp-44, Glu-45, Asp-128, and Asp-135 were also tested (see Fig. 1).

As reviewed by Colquhoun (22), when comparing two receptors, the information obtained from maximum responses is limited by the existence of a correlation with the number of functional receptors and by errors that result from desensitization. In the present study the first concern is addressed by the use of a-Bgt binding to determine the amount of receptors present at the oocyte membrane and correlate the currents produced by them. Sucrose gradient sedimentation experiments of relevant mutant receptors (data not shown) indicate that they are in the typical pentameric form, and we assume that the detected binding sites correspond to well folded receptors, although there is no guarantee that all receptors so measured are functional. In this sense, a general conclusion is that all mutants, except K46A and K46R, showed a substantial decrease in receptor expression, suggesting that tight structural requirements for folding, assembly, and/or traffic exist in loops 2 and 7. Despite strong reductions in receptor expression, the amount of receptors present at the oocyte membrane was sufficient to detect measurable currents; consider, for instance, a typical amount of 1.5 fmol of bound α-Bgt/oocyte and an I\textsubscript{max} of 1 μA (1 mm ACh and holding potential −80 mV) for a mutant expressing only 15% of the control α\textsubscript{7} wild-type receptors.

The other important question is whether the peak responses are affected differently by factors such as desensitization or channel block at the high agonist concentrations used. Concerning this point several data argue against a selective effect of the mutations on these processes: (a) all currents evoked upon continuous stimulation with high agonist concentrations decreased with the same time course in wild-type and mutant receptors (data not shown), and (b) mutations at the same positions in loops 2 and 7 have been shown not to affect desensitization in muscle nicotinic receptors (25). Although we cannot absolutely rule out an effect of the mutations on very fast desensitization, which was out of reach of our experimental setup, in the present study we have considered maximal responses as an efficient indicator of gating mechanisms (see Ref. 22 for an ample discussion on the binding-gating problem).

Mutants of the positively charged residue Lys-46 but not Arg-130 (for instance, mutants K46A and K46E) appeared to have altered gating mechanisms unless a positive charge was present (K46R). Therefore, a pairwise electrostatic interaction between Asp-266 and Lys-46 appeared plausible, and in this case the double charge reversal mutant K46D/D266K should have its function restored. However, it was not possible to test its activity because no mutant receptors were expressed. The cysteine cross-linking approach also was not possible with mutant K46C/D266C because of the same problem. Then we tried an alternative approach to this issue with co-injection experiments despite the impossibility of knowing exactly how many wild and mutant subunits conform a receptor pentamer. From the proportions of the RNAs used for co-injections (1:4, wild to mutant subunits) one should expect that mutant subunits are components of these mixed receptors, and some data favor this assumption; if pentamers were only made of wild-type subunits because mutant subunits are excluded from this process, then the decrease in expression observed when mutant subunits are co-injected should not occur. However, it could be argued that mutant subunits are not excluded from this process but act as dominant negative mutants, inducing receptor degradation and consequently the mentioned reduction in receptor expression. In any case, the resultant receptors would be made of only wild-type subunits. However, this does not seem to be the case.
to be the case because these receptors showed different EC_{50} values for two different agonists (Fig. 6C) and smaller currents than expected from their expression levels (Fig. 6B). In fact, these properties, different from the ones observed with wild-type receptors, must be the outcome of the incorporation of mutant subunits into the pentamers. If this is true, and whatever their stoichiometry in them, the double charge reversal mutants cannot mimic the behavior of wild-type α7 subunits given the differences mentioned above. In contrast with GABAR, a gating mechanism based solely on the electrostatic interaction between Lys-46 and Asp-266 does not seem to be operative in α7 nAChRs.

A wide network of interactions would then be possible as polar and charged residues not only have pairwise interactions in proteins but also form structurally and functionally relevant networks able to link distant domains in proteins (23, 24). Different residues, including Glu-45, Lys-46, Asp-128, and Asp-135, would participate in this network although with diverse requirements and roles. Thus, Glu-45 and Asp-135 do not even admit conservative mutations because mutants showed reduced or no functional responses in all cases. Glu-45 is present in all nAChR and GlyR subunits as well as in the β subunits of the GABAR. In addition, the homologous residue Glu-53 in GlyRs (Fig. 8) was also shown to affect channel gating (14). Therefore, it is an attractive candidate to form part of a global gating mechanism of this superfamily. Regarding Asp-135, it may play a pivotal role because this amino acid is present at homologous locations in all subunits of this gene superfamily. Moreover, homologous residues Asp-148 in GlyRs and Asp-149 in GABA_{R}s (Fig. 8) have been found to be implicated in channel gating (12–14, 26) suggesting that independently of which charged amino acid is present at the M2-M3 linker the presence of an aspartate residue at this location of loop 7 is the essential. Recently, the gating parameters of many mutants of residues at loops 2 and 7 of the α1 nAChR have been reported (25). Whereas Glu-45 was not studied, the only analogous mutant of Asp-135 able to produce currents (D135E) did not show large differences in gating when compared with wild-type nAChRs, precluding a clear conclusion about the involvement of this amino acid on the gating mechanism of muscle type nAChRs. Notice, however, that in these type of mutant receptors, three subunits (γ, δ, and ε) would still have an aspartate at the mentioned position and therefore could contribute to the hypothetical network.

Another class of residues is formed by Lys-46 and Asp-128, which tolerate exchange by other amino acids with the same charge. Lys-46 is also present in α2, α4, β1, and ε nAChR subunits and substituted by Arg in β2, β4, and γ subunits. It is absent only in the α1, α3, and δ subunits, although they must assemble with other subunits that have this positively charged residue. Regarding other ligand-gated ion channels, Lys-46 is also present in 5-HT_{3} receptors but absent in all glycine and GABA_{R} receptor subunits. Therefore, this amino acid could be a good candidate to integrate the gating mechanism in cationic but not anionic receptors. Miyazawa et al. (10) suggested that the hydrophobic residue α1V46, which is positionally equivalent to α7K46, could participate in the interaction with the M2-M3 linker that drives channel gating. Several mutants of α1V46 have been shown to have their gating properties modified (25) suggesting that this position is important for the gating mechanism and confirming that this mechanism does not rely entirely on electrostatic interactions.

Finally, mutants of Asp-128 (D128A and D128E) had larger functional responses (3- and 2-fold larger, respectively) than expected from their expression levels. Normally, a gain of function effect should produce an increase in agonist potency. Interestingly, D128A showed pharmacological properties similar to wild-type receptor, suggesting a complex behavior that we could not explain through simple kinetic schemes. Unfortunately, Chakrapani et al. (25) did not include in their exhaustive study the homologous residue α1I130.

Auerbach and co-workers (27, 28) proposed a gradual series of transitions or “conformational wave” for the gating event. They also demonstrated that a negatively charged residue, Asp-97 of the nAChR α1 subunit, is an early link in the AChR gating reaction (29), which is probably followed by movements of the loop2/loop 7 domain (25). The amino acid residues that we have shown to affect the gating process are located in these loops and should integrate a mechanism able to transmit this conformational wave from the agonist binding site to the M2-M3 linker. The conformational wave would be the result of different interactions generated not only by ionic bonds but also by charge-dipole interactions, hydrogen bonds, and other types of interactions (30). In this way, a particular ligand-gated channel might show specific and perhaps subtle differences within a general gating mechanism.

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