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# A single residue in the M2-M3 loop is a major determinant of coupling between binding and gating in neuronal nicotinic receptors

**(acetylcholine receptor/site-directed mutagenesis)**

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**ABSTRACT** **Binding of agonists to nicotinic acetylcholine receptors generates a sequence of changes that activate a cation-selective conductance. By measuring electrophysiological responses in chimeric α7/α3 receptors expressed in *Xenopus* oocytes, we have showed the involvement of the M2–M3 loop in coupling agonist binding to the channel gate. An aspartate residue therein, Asp-266 in the α7 subunit, was identified by site-directed mutagenesis as crucial, since mutants at this position exhibited very poor functional responses to three different nicotinic agonists. We have extended this investigation to another neuronal nicotinic receptor (α3/β4), and found that a homologous residue in the β4 subunit, Asp-268, played a similar role in coupling. These findings are consistent with a hypothesis that the aspartate residue in the M2–M3 loop, which is conserved in all homomer-forming α-type subunits and all neuronal β-type subunits that combine to form functional receptors, is a major determinant of information transmission from binding site to channel gate in all neuronal nicotinic receptors.**

The nicotinic acetylcholine (ACh) receptor (AChR) is a member of the superfamily of ligand-gated ion channels (LGIC) that is found in the synaptic membranes of nerve, muscle, and electric organ cells (1–3). It is assumed that the binding of two molecules of agonist to the AChR opens the channel, activating a cation-selective current. AChRs are good examples of allosteric proteins, as the signal generated at the binding-site region must be transmitted to the channel gate located some distance away, and this involves a more than local conformational change. Electron micrograph studies have recently suggested that ACh triggers localized disturbances at the binding site of AChRs, which are communicated to the gate in the membrane via small rotations of the subunits (4). Residues near the M2 segment could take part in transmission of the signal (4, 5). Recently, residues located in the extracellular M2–M3 loop have been reported to affect coupling in homomeric glycinergic receptors (6, 7) and γ-aminobutyric acid (GABA) receptors (8), but studies on this region of AChRs have not been reported. Identification of such residues is of interest because it can provide insights into the allosteric changes that take place during activation of LGICs. We report here that mutations of an homologous single residue in the M2–M3 loop of two different neuronal nicotinic receptors (α7 and α3/β4) caused dramatic reductions in the maximal currents evoked by three different nicotinic agonists, suggesting that this residue is critical for the transduction of agonist binding into channel activation of neuronal nicotinic AChRs receptors.

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## MATERIALS AND METHODS

**Construction of Chimeras and Site-Directed Mutagenesis.** Chimeras and point mutations were made by performing two successive PCR amplifications as described (9, 10) and were confirmed by sequencing the cDNA clones.

**Oocyte Expression.** Chimeric or mutated DNAs were inserted into the pSP64T vector (11). Capped mRNA was synthesized *in vitro* using SP6 RNA polymerase. Defoliculated *Xenopus* oocytes were injected with 5 ng of RNA in 50 nl of sterile water, and measurements were made within 3–6 days after injection.

**Electrophysiological Recordings.** Oocyte whole membrane currents were measured with a two-electrode voltage-clamp amplifier OC-725B (Warner) at room temperature (22–25°C). To avoid the presence of contaminating calcium-activated chloride currents, all oocytes were incubated for 3–4 h in Barth's storage media containing 100 μM of the membrane permeant form of the calcium-chelator bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA; Texas Fluorescence Laboratory, Austin). After this, we found no evidence of secondary chloride currents, as no biphasic currents were detected upon cholinergic stimulation at any holding potential (12). Oocytes were perfused with frog Ringer's solution containing 135 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM Hepes (pH 7.2). Nicotinic agonists [acetylcholine, dimethylphenylpiperazinium (DMPP), and (−)-nicotine, purchased from Sigma] were applied through a 1.5-mm diameter pipette located close to the oocyte. Currents were filtered at 20 Hz by an 8-pole Bessel filter, digitized at 250 Hz, and stored on hard disk for later analysis. Data were acquired by a DigiData 1200 interface driven by PCLAMP software (Axon Instruments, Foster City, CA).

**[$\alpha^{125}$I]Bungarotoxin (α-Bgt) Binding Assays.** Total surface expression of α-Bgt (Amersham) binding sites was tested with 5 nM α-Bgt as described (10). For experiments with mutated α7 subunits in which normalized ionic current data were required, binding assays were performed in individual oocytes.

**Data Analysis.** Current amplitudes were measured at the peak inward current, and no correction for desensitization was

*Abbreviations*: ACh, acetylcholine; AChR, ACh receptor; LGIC, ligand-gated ion channel; α-Bgt, [$\alpha^{125}$I]bungarotoxin; GlyR, glycinergic receptor; DMPP, dimethylphenylpiperazinium; A263S, Ala-263 → Ser mutation in α7 subunit; D266L, Asp-266 → Leu mutation in α7 subunit; D266N, Asp-266 → Asn mutation in α7 subunit; D268A, Asp-268 → Ala mutation in β4 subunit; WT, wild type; GABA, γ-aminobutyric acid.

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made. Dose-response curves were fitted using a nonlinear least squares algorithm to the Hill equation: $I/I_{max} = 1/[1 + (EC_{50}/C)^h]$, where $EC_{50}$ is the agonist concentration that elicits the half-maximal response, h is the Hill coefficient, and $C$ is the agonist concentration. Statistical significance was calculated by Student $t$ test or nonparametric Mann–Whitney test.

## RESULTS AND DISCUSSION

**Studies on Chimeric Receptors.** The use of chimeric protein constructs has been helpful in understanding the structure and function of many components of the transduction pathway in LGICs (13–15). Subunits that are able to form homomeric functional ionic channels are particularly useful for such a purpose. In a previous paper (10), a chimeric construct of α7 and α3 subunits, named C5 (see Fig. 1), was shown to be able to bind nicotinic ligands but not to allow ionic currents. It was suggested that coupling between agonist binding and opening of the channel was somewhat interrupted, as had been suggested for other chimeric constructs (15). We have tried to localize structural domains that could be involved in the coupling between the ligand binding site and the gate that controls the ionic pore of neuronal nicotinic receptors. Thus, the strategy of extending the α7 domain in C5 was followed in order to restore the ability of the channel to open.

Fig. 1 shows different chimeric constructs containing the N-terminal part of the α7 subunit and the complementary C-terminal domain of the α3 subunit. Starting on C5, inclusion of the putative pore-lining segment, M2, and the extracellular loop M2–M3 of the α7 subunit, as in construct C10, restored the ionic current evoked by 50 μM nicotine. Next, other chimeras were constructed, where the junctions of the domains of the two different α subunits were located between those of C5 and C10. Constructs C12 and C13 did bind α-Bgt, but no ionic currents were detected upon nicotinic stimulation. In contrast, constructs C20 and C21 showed functional responses.

**Studies on Point Mutations.** Although there were some interesting differences in the extent of functional responses to 50 μM nicotine among the constructs (compare binding and current figures of C10, C20, and C21), we directed our attention to the most dramatic difference: C20 allowed current flow and C13 did not. When sequences of these two constructs were compared, they were found to differ only in two residues, Ala-263 and Asp-266 in C20, which were Ser and Leu, respectively, in C13. Thus, these two residues could have been responsible of the dramatic difference observed in their functional response. These two residues were mutated separately in wild-type (WT) α7 subunits and the resultant receptors were challenged with 50 μM nicotine. Fig. 2 shows that Ala-263 → Ser (A263S) α7 receptors seem to have a channel-gating mechanism comparable to that of WT, as nicotine is able to evoke large ionic currents. In sharp contrast, nicotine evoked hardly any detectable currents in mutant Asp-266 → Leu (D266L). A more conservative mutant, Asp-266 → Asn (D266N), showed very small currents upon stimulation with 50 μM nicotine (note the different calibration bars).

The tiny functional responses in α7–Asp-266 mutants could be explained by at least one of the following changes: (*i*) reduced expression of receptors; (*ii*) there might be a mixture of functional and nonfunctional receptors on the cell surface,

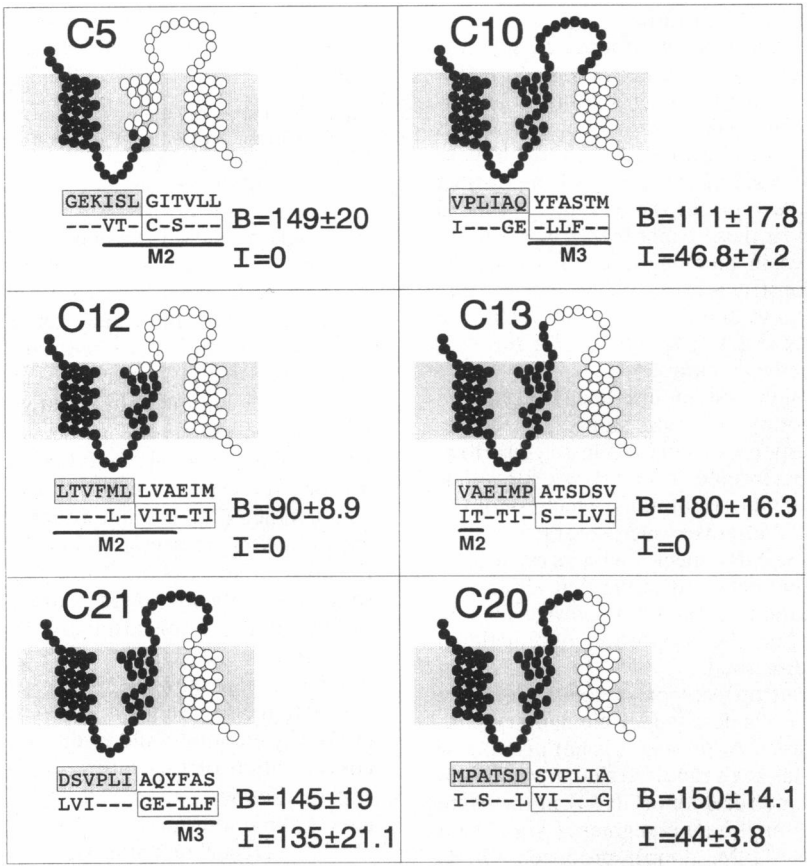


FIG. 1. Schematic diagrams and functional expression of α3/α7 chimeras. Putative transmembrane segments M1, M2, and M3 and loops connecting them are displayed with circles representing individual amino acids (α3, ○; α7, ●). The residues of the area where the α3 and α7 domains joint are also displayed (α3, bottom; α7, top), and two boxes comprise the actual sequence of the construct (α3, transparent; α7, shaded). Expression was tested by α-Bgt binding and nicotine-evoked ionic currents (50 μM). All data were normalized to the arithmetic mean of those obtained with the α7 subunit alone, and means ± SEM of values obtained from at least 30 oocytes (3–5 donors) are shown. Typical control values for α7 subunit were 2–5 fmol of bound α-Bgt/oocyte, and 1–3 μA/oocyte at −40 mV.

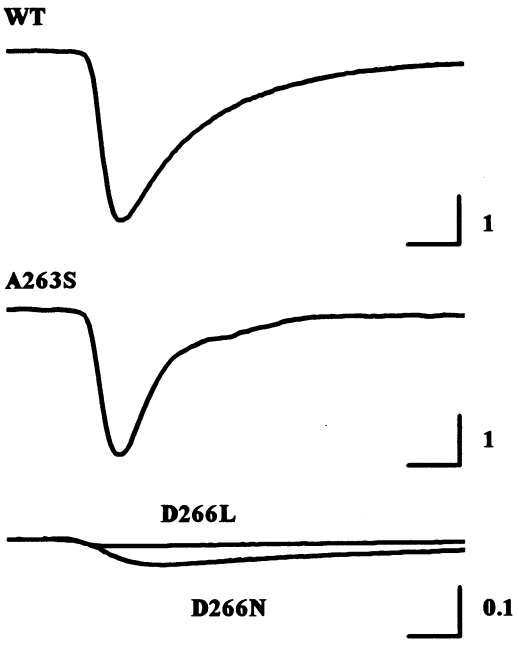


FIG. 2. Inward currents obtained upon stimulation with 50 $\mu$M nicotine in oocytes expressing $\alpha$7 receptors. Holding potential was $-80$ mV. Horizontal calibration bars are 100 ms. Vertical calibration bars are in $\mu$A.

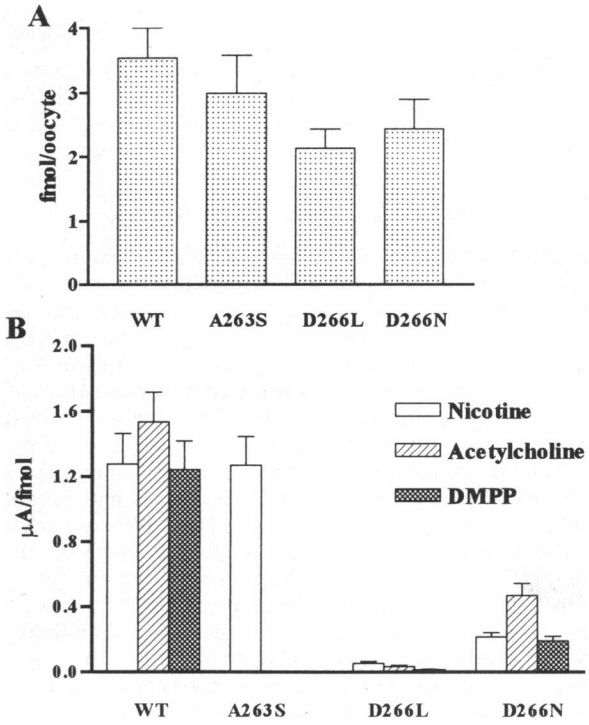


FIG. 3. (*A*) Surface expression of $\alpha$-Bgt binding sites. Data are means ± SEM of 30–38 individually measured oocytes from four donors. Surface binding sites in both $\alpha$7–Asp-266 mutants, but not A263 mutant, were significantly smaller than those in WT $\alpha$7 receptors (unpaired *t* tests, $P = 0.02$). (*B*) Mean maximal currents ± SEM upon stimulation with nicotinic agonists. Inward currents were obtained at $-80$ mV (250 $\mu$M for WT and A263S, and 3 mM nicotine for $\alpha$7–Asp-266 mutants; 3 mM ACh; 1 mM DMPP), then divided by the value obtained in the binding measurement for the same oocyte, as in *A*. Normalized currents in $\alpha$7–Asp-266 mutants were significantly smaller than those in WT $\alpha$7 receptors (unpaired *t* tests, $P < 0.0001$). ACh and DMPP were not tested in A263S receptors.

both of which can bind $\alpha$-Bgt; (*iii*) impaired binding; (*iv*) impaired gating; and (*v*) reduced single channel conductance. These possibilities are considered in turn.

(*i*) Expression of surface receptors with mutated subunits could have been greatly reduced. This would happen if, for example, these residues were important in folding or assembling processes. Fig. 3*A* shows surface expression of receptors as measured by binding of radiolabeled $\alpha$-Bgt. Although there are some differences in the level of expression of the distinct constructs, they could not account for the extremely reduced ionotropic response. Prior to these experiments, we demonstrated that the differences in binding were not caused by changes in affinity of the constructs for $\alpha$-Bgt, by showing that all receptors, WT, and mutants, bound $\alpha$-Bgt with roughly the same affinity ($K_d$ values were 2.3, 2.1, and 1.7 nM for WT, D266N, and D266L, respectively; data not shown).

(*ii*) The oocyte expression system has been questioned because it yields some nicotinic receptors showing heterogeneity in their functional responses (reviewed in ref. 3). However, those studies were performed in muscle AChRs, thus involving more than one subtype of subunits, which is not the case in the present report. Moreover, sucrose gradient centrifugation gave unique molecular species with sizes close to that of *Torpedo* AChR monomers, indicating that all mutant receptors assembled in pentamers (data not shown). Though an effect of the mutation on the proportion of functional receptors cannot be fully discarded.

(*iii*) The reduction of ionotropic responses could have been due to modifications in the single channel elementary conductance of mutated receptors. At present it is not possible to rule out this possibility. It has been reported that mutations of homomeric human $\alpha$1 glycine receptors (GlyRs) in the same area, but closer to the M2 lining channel segment (Arg-271 on the putative extracellular M2–M3 loop), produced GlyRs which displayed very different single channel properties compared to the native receptors (6, 7, 16, 17). GlyRs display multiple conductance states and single channel data have demonstrated that distributions of conductance states in mutant GlyRs contribute significantly to the reduction in glycine-activated currents in cells expressing those mutants (6, 16). Although measurements of $\alpha$7 single channel events have been elusive (18), there is no evidence in favor of the existence of multiple conductance states in $\alpha$7 receptors (19). Different conductive states have been reported for neuronal nicotinic receptors, but they have been attributed to variable stoichiometry, which could not be the case in $\alpha$7 homomers (20). As the mutation eliminated a negative charge, it could be argued that the observed effect was due to a change in cation permeability. Though mutations of residues thought to be forming the external mouth of the pore affect single channel conductance (21, 22), mutations located somewhat closer to the putative pore region than $\alpha$7–266-Asp, have not affected the pore properties (22). Moreover, if this residue were part of some selectivity filter on the extracellular side, then its removal would not affect outward currents, yet the same reduction of current flow in mutant $\alpha$7 receptors was found in both directions (not shown).

(*iv*) If the binding was impaired, then the small currents evoked by nicotine could be due to a wrong choice of either the concentration or the nature of the agonist. This alternative explanation was addressed by carrying out dose-response curves of nicotinic agonists. $EC_{50}$ values of nicotine, ACh, and DMPP in D266N receptors were 4- to 7-fold higher than in WT receptors (Fig. 4). This suggests that, despite the capacity of agonists to bind to the receptor, a small change in their affinity could have been occurred (see discussion below), and this could explain, at least in part, the reduced responses to 50 $\mu$M nicotine. Dose-response relationships in D266L receptors were not produced because their ionic currents could not be

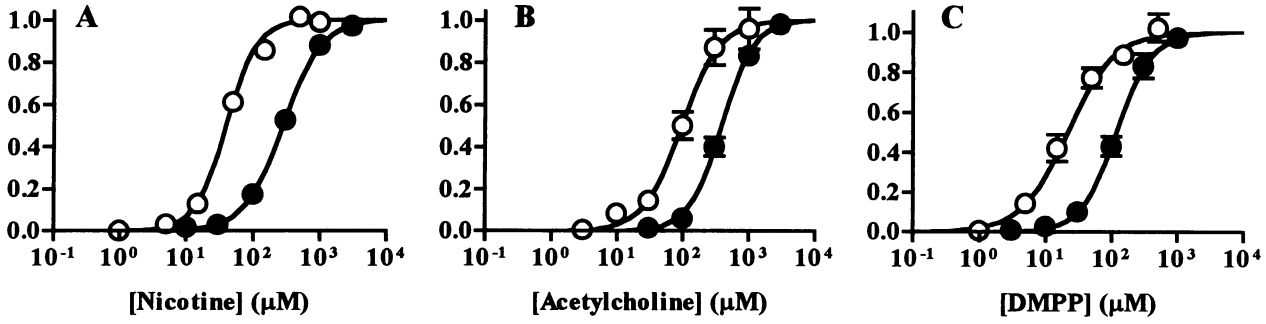


FIG. 4. Dose-response curves of oocytes expressing α7 WT (○) and D266N receptors (●). Data points represent the mean ± SEM. Continuous lines represent fits of data to the Hill equation. (*A*) Nicotine (11 oocytes, 3 donors); $EC_{50}$ values of 41 ± 3 and 276 ± 5 μM, and Hill coefficients of 1.72 and 1.54, for WT and D266N receptors, respectively. (*B*) ACh (5–9 oocytes, 2–3 donors); $EC_{50}$ values of 96 ± 11 and 385 ± 25 μM, and Hill coefficients of 1.51 and 1.79, for WT and D266N receptors, respectively. (*C*) DMPP (4–10 oocytes, 2–3 donors); $EC_{50}$ values of 21 ± 2 and 117 ± 13 μM, and Hill coefficients of 1.25 and 1.63, for WT and D266N receptors, respectively.

accurately measured, however agonists were able to displace α-Bgt (not shown).

(*v*) Finally, to discard possible effects of diminished expression and lower affinities, we used a concentration of agonists that gave maximal ionic currents and also normalized the magnitude of these currents to the concentration of receptors present at the oocyte surface. Fig. 3*B* shows that, even after these corrections, α7–Asp-266 mutants exhibited very poor functional responses to the three agonists. In contrast, ionotropic responses of A263S receptors were identical in magnitude to those of WT receptors.

An important question is whether at the high agonist concentrations used the peak current is a genuine measure of maximal responses, or whether it is too affected by other factors (desensitization or channel block) to allow conclusions to be drawn. It is unlikely that the reduction of peak responses in mutants was due to a selective effect of the mutation on desensitization or channel block because currents evoked upon continuous stimulation decrease with the same time course in WT and mutant receptors (not shown).

Assuming that there is no change in either the elementary conductance or the proportion of functional channels, and according to the most accepted kinetic schemes, the substantial decreases of maximal responses could only be explained in terms of altered kinetics after binding of the three agonists—i.e., coupling or gating. In agreement with this idea, mutations at the analogous position in GABA ρ1 receptors have been reported to affect gating (8). Similarly, mutations of human α1 GlyRs have showed that taurine and β-alanine switched from agonists to competitive antagonists with only slight changes in affinity (17), and picrotoxin, a competitive antagonist of native GlyRs, behaved as an allosteric potentiator in mutant GlyRs (16).

**Possible Effects on Agonist Binding.** A question that could be addressed is whether changes in coupling or gating alone could account for the higher $EC_{50}$ values found in mutant receptors. In addition to the well-established role of cysteine residues and the extracellular disulfide loop on cholinergic binding (23), other residues near the extracellular side of transmembrane segments M1 and M2—i.e., N-terminal region and M2–M3 loop—have been involved in binding of agonists to LGICs (16, 17, 24–28). It is possible that α7–Asp-266 is also involved in binding of nicotinic agonists to neuronal nicotinic receptors. Fluorescence studies and electron images have determined that the ligand binding site of muscle AChRs is located 25–35 Å above the membrane surface (4, 29). This distance is too great to consider it likely that the mutated residues were constituent of the ligand binding site, but the possibility of some long-distance effect on its structure cannot be fully discarded. Whatever the case, the results presented here show that changes in $EC_{50}$ are not large (less than one order of magnitude in α7-D266N receptors). Using a conventional kinetic scheme of five states, as described in Franke *et al.* (30), which mimicked the main features of whole-cell currents flowing through α7 receptor channels, we found that, mostly because of the presence of desensitization, decreasing the maximal response by 80% with respect to WT (as in D266N) could only account for a 3-fold change in $EC_{50}$ (4- to 7-fold changes shown in Fig. 4). For these α7 mutants, how-

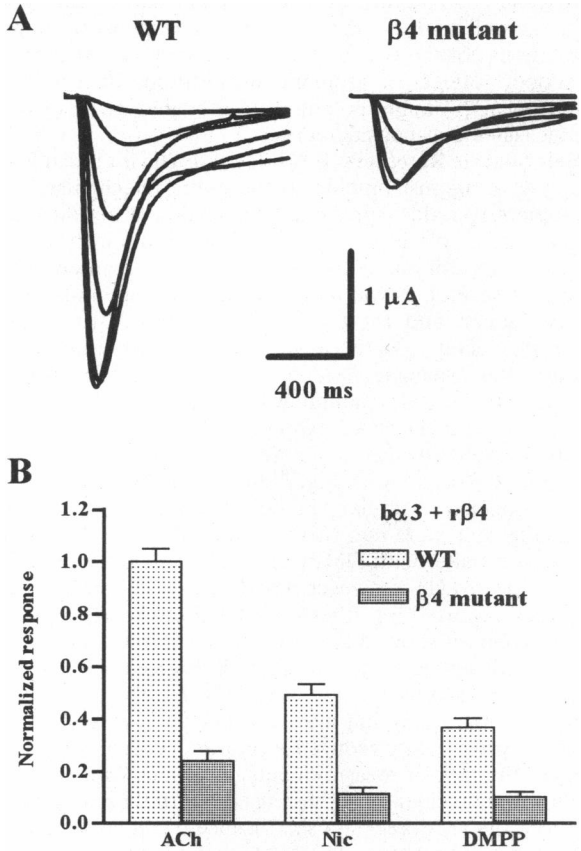


FIG. 5. Expression of heteromeric neuronal nicotinic receptors. (*A*) Inward currents obtained upon stimulation with ACh of α3/β4 WT and α3/β4-D268A receptors. Traces represent currents obtained at −80 mV with 10, 30, 100, 300, 1000, and 3000 μM ACh, from top to bottom, respectively. (*B*) Maximal currents were obtained after challenge with ACh (3 mM), nicotine (1 mM), and DMPP (1 mM). Currents were normalized to the mean ACh-evoked current obtained in WT receptors for each batch (four donors). Data are means ± SEM for oocytes expressing WT ($n = 33$) and mutated ($n = 37$) β4 subunits. Currents in mutated receptors were significantly smaller than those in WT receptors for all agonists (unpaired Mann–Whitney test, $P < 0.0001$).

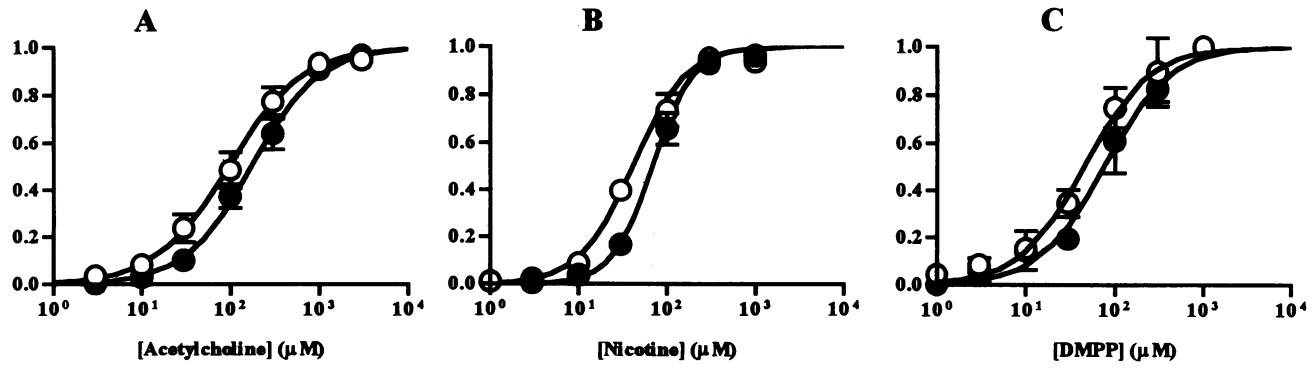


FIG. 6. Dose-response curves of oocytes expressing WT (○) and mutated heteromeric receptors (●). Data points represent the mean ± SEM. Continuous lines represent fits of data to the Hill equation. (*A*) ACh (11 oocytes, 3 donors); $EC_{50}$ values of 96 ± 17 and 173 ± 30 μM, and Hill coefficients of 1.06 and 1.16, for WT and mutant β4-containing receptors, respectively. (*B*) Nicotine (5 oocytes, 2 donors); $EC_{50}$ values of 41 ± 4 and 68 ± 5 μM, and Hill coefficients of 1.47 and 1.92, for WT and mutant β4-containing receptors, respectively. (*C*) DMPP (3 oocytes, 1 donor); $EC_{50}$ values of 45 ± 10 and 75 ± 18 μM, and Hill coefficients of 1.20 and 1.21, for WT and mutant β4-containing receptors, respectively.

ever, an extended kinetic scheme with an additional closed state, located between the doubly-occupied closed and the open receptor, would account for the slightly larger-than-predicted shifts in $EC_{50}$. The forward rate constant communicating the two doubly occupied closed states might be lower in α7–Asp-266 mutants, explaining quantitatively the shift in $EC_{50}$ as well as the reduced maximal response (computer simulations not shown). Single channel data recently reported to support effects of aromatic substitutions on cholinergic binding (25, 27) might be reinterpreted by using an extended kinetic scheme as considered here.

**Heteromeric Receptors.** If residue Asp-266 is crucial for the coupling of agonist binding to the gating mechanism in α7 homomers, it could be presumed that analogous residues could play a similar role in other nicotinic receptors with different subunit composition. With respect to this, it is interesting to note that several β-type neuronal nicotinic subunits, for instance human and rat β4 and β2 subunits, also have an aspartate residue located at an equivalent position. To test whether this aspartate residue was also important in β-type subunits, the rat β4 subunit was mutated at this location, Asp-268 → Ala (D268A), expressed in combination with the bovine α3 subunit; the results were compared with the combination of WT subunits. Fig. 5 shows that ACh-evoked ionic currents displayed similar kinetics for both subunit compositions (Fig. 5*A*). Note that currents showed desensitization and also were smaller with DMPP (Fig. 5*B*), in agreement with a recent report (31). However, nondesensitizing currents have also been reported for other α3/β4 receptors (32, 33). Dose-response curves showed that receptors with mutated subunits have slightly higher $EC_{50}$ values for all three nicotinic agonists (values increased by a factor of 1.7; Fig. 6). The most striking difference was again that maximal functional responses to all agonists were greatly reduced in mutant receptors (Fig. 5*B*). Despite the lack of measurements of surface expression, the qualitative coincidence of these results with those obtained in α7 receptors reinforces the significance of the aspartate residue in this position of the M2–M3 loop in the kinetic processes, leading to the opening of the ionic pore with only small, if any, effects on agonist binding.

**Possible Mechanisms.** Mutations have eliminated a negative charge, therefore a likely mechanism for the reduction in functional responses could be the absence of the regulatory role of calcium described in neuronal AChRs (34, 35). We have found that changes in external calcium concentration affected equally both WT and mutants (α7-D266N and α3/β4-D268A), ruling out a possible role of calcium in the reduction of responses (data not shown). In fact, the location of the external regulatory site for calcium has been postulated to be at the large N-terminal extracellular domain (15), which is common for all mutants tested.

It has been suggested that signal transmission from the binding site to the pore is probably mediated by transference of electrons and/or conformational changes located at or near the segment M1 and/or M2–M3 loop (4, 5). Residues involved in this pathway could be aromatic or electrically charged, and so α7–Asp-266 and β4–Asp-268 are good candidates to play a role in such a transmission mechanism. Unwin (4) has recently suggested that cholinergic agonists trigger localized disturbances at the binding site of AChRs, whose effects are communicated to the structure in the membrane through small rotations of the subunits. Finally, such rotations are transmitted to the gate, creating a weakly stabilized open pore. Since α7–Asp-266 and β4–Asp-268 are probably located in the proposed shaft between the binding-site region and the membrane-spanning portion of the receptor, it seems consistent to suggest that they facilitate the aforementioned rotations. In contrast, mutated receptors would impair the rotations of some or all subunits, thus stabilizing them in doubly occupied closed states.

This paper reports the finding that neuronal AChRs mutated at a single residue in the M2–M3 loop display poor functional responses, suggesting an impaired coupling between the binding of a number of agonists and the gating of the ionic channel. Because the aspartate residue in the M2–M3 loop is conserved in all homomer-forming α-type subunits and all neuronal β-type subunits that combine to form functional receptors, it is strongly suggested that this single residue is crucial for the coupling of the signal generated at the binding site to the channel gate in all neuronal nicotinic receptors. However, single channel experiments are needed to discard pore effects of the mutations and to elucidate a more exact picture of the events taking place halfway between the binding of agonists and the opening of the channel.

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