Desensitization of Neuronal Nicotinic Acetylcholine Receptors Conferred by N-Terminal Segments of the β2 Subunit†

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Received August 23, 2000; Revised Manuscript Received November 27, 2000

ABSTRACT: Desensitization is a general property of ligand-gated ion channels. Because of a wide array of available subunit combinations, it generates different time constants for channel closure, thereby modulating the processing of information in the brain. Within the family of neuronal nicotinic acetylcholine receptors (nAChRs), α3β2 and α3β4 receptors display contrasting properties of desensitization. When measured using two-electrode voltage-clamp in *Xenopus* oocytes, desensitization results in current decreases 2 s after initiation of acetylcholine application by 94% for α3β2 receptors, but only by 6% in the case of α3β4 receptors. Desensitization was analyzed by inserting different portions of the β2 into the β4 subunit. Residues 1−212 of the β2 subunit were able to confer 78% desensitization in 2 s, while smaller chimeras revealed desensitization in 2 s conferred by residues 1−42 alone to a level of 50%, by residues 72−89 to a level of 74%, and by residues 96−212 to a level of 77%. Some long-term (25 min) effects of desensitization driven by acetylcholine were found to rely partially on the same elements, including an enhancement mediated by residues 1−95 and 96−212 of the β2 subunit individually. Our results reveal that desensitization relies independently on diverse portions of the extracellular domain of the β2 subunit. Phenotype of α3β4 involves, in contrast, complex structural requirements involving residues dispersed throughout the entire N-terminal domain of the β4 subunit.

Nicotinic acetylcholine receptors (nAChRs)[1] are pentameric transmembrane allosteric proteins involved in cholinergic transmission at the neuromuscular junction and in the brain (*1*, *2*). In response to agonist exposure, they undergo two types of transitions: the first is a rapid (ms) opening of the channels, resulting in membrane depolarization; the second is a slower (s) progressive decline of the current response, corresponding to the stabilization of nAChRs in refractory closed forms called desensitized states. Desensitization includes fast and slow components that have been observed both in vivo by electrophysiological recordings (*3*, *4*) and in vitro with membrane bound or reconstituted receptors by rapid chemical methods (*5*−*7*).

Desensitization is of particular interest in the case of neuronal nAChRs, since it may regulate the activity of neurons in the brain in conditions where cholinergic ligands are present over large time periods (*8*). Given the pentameric nature of nAChRs, a high diversity of desensitization features is provided by the various neuronal nicotinic subunits cloned [9 α-subunits (α2−α10) and 3 β-subunits (β2−β4)]. A specific role is attributed to the β2 and β4 subunits that contribute to the binding pocket at the subunit interface by three loops of amino acids facing the adjacent α-subunit (*9*). These subunits potentially account for some pharmacological differences of nAChRs (*10*, *11*), as well as for the differences in desensitization observed, for instance, between the α3β2 and the α3β4 receptors (*12*).

The α3β2 and α3β4 receptors are differentially distributed throughout the nervous system. The α3β2 receptors are present in many areas of the brain where they were proposed to promote the release of other neurotransmitters, for example, in dopaminergic pathways (*13*), while α3β4 receptors are typically expressed in ganglia but also in the medial habenula (*14*, *15*). In locations where both β2 and β4 subunits may coexist within the same receptor oligomer, such as in the medial habenula (*16*), the precise stoichiometry of subunits in the receptor assembly may influence the rate of desensitization and the processing of information.

It therefore appeared of interest to gain insight into the structures that underlie differences in desensitization between α3β2 and α3β4 nAChRs. In a series of chimeras produced to delineate which portions of β2 or β4 subunits account for these characteristics, both short-term kinetics (15 s recordings) and levels of desensitization resulting from 25 min acetylcholine (ACh) exposure of α3β2 and α3β4 receptors were investigated in *Xenopus* oocytes. Our results provide evidence for an essential contribution of the extracellular, N-terminal domain of the β-subunit in desensitization kinet-

† This work was supported by research grants to J.P.C. from the Fondation Gilbert Lagrue, the Association Française contre les Myopathies, the Collège de France, the EEC Biotech and Biomed Programs, the Council for Tobacco Research, the Reynolds Pharmaceuticals, the Délégation Générale de l'Armement, and the Swiss Office Fédéral de l'Education et des Sciences, and to Daniel Bertrand from the Swiss National Science Foundation.

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptors; ACh, acetylcholine.

10.1021/bi0020022 CCC: $20.00
Published on Web 01/24/2001

ics as well as steady-state levels of desensitization. Desensitization of the β4-containing receptor can be accelerated by multiple elements of the N-terminal domain of the β2 subunit; conversely, in the β2-containing receptor, desensitization can be decreased only by substitution of the β4 N-terminal domain (residues 1−212).

MATERIALS AND METHODS

Construction of Chimeras between β2 and β4 Subunits. cDNAs encoding the β2 and the β4 rat subunits were excised as a *Not*I/*Xho*I fragment from the clones kindly supplied by Dr. J. Boulter and introduced into the pMT3 vector for expression in *Xenopus* oocytes (*9*). Chimeric constructs were obtained either using common restriction sites (*Bbs*I site at position 212) or introducing new restriction sites by site-directed mutagenesis using the Quickchange Mutagenesis kit supplied by Stratagene (*Bsr*GI site at position 95 in β2, *Hpa*I site at position 46 in both β2 and β4). Transfer of smaller cassettes (residues 72−89, 77−81) was performed by direct mutagenesis of entire stretches of residues.

Electrophysiological Recordings and Desensitization Measurements. Xenopus oocytes were prepared, injected, and recorded as previously described (*17*). Whole cell recordings were performed in OR_2 medium (containing 82.5 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 15 mM HEPES, pH 7.4) at 18 °C, using a Geneclamp amplifier from Axon Instruments, Forster City, USA. Holding potential was −100 mV, and flow rate was 6 mL/min.

For desensitization characterization, currents were evoked by saturating concentrations of ACh (500−1000 μM) and measured both at peak amplitude (maximally evoked currents I_m) and 2 s after the peak amplitude (I_{2s}). Ratio D was computed as $D = (I_m - I_{2s})/I_m$ and used to characterize the extent of desensitization.

Fitting and Modeling. Currents evoked by various concentrations of ACh (2−1000 μM) were normalized to their maximal value and fitted by the equation:

$$i_0 = i_f \exp(-t/\tau_f) + i_s \exp(-t/\tau_s) + i_r \qquad (1)$$

that includes a fast component i_f and a slow component i_s, with corresponding time constants τ_f and τ_s, and a residual current i_r. This equation accounts for the biphasic nature of desensitization, where the fast component i_f is viewed as giving rise to the intermediate desensitized state I (*3*, *4*) and the slow component giving rise to the final desensitized state D (*18*−*24*). It provides satisfactory agreement between theoretical curves and experimental traces (correlation >0.99 in most cases), whereas single exponential fitting results in visible discrepancies at the break between the fast and the slow components. The amplitude of fast and slow components is found to differ strikingly between α3β2 and α3β4 receptors, whereas time constants τ_f and τ_s do not show significant variation.

	i_f	i_s	τ_f (s)	τ_s (s)
α3β4	6	94	0.93 ± 0.16	150 ± 86
α3β2	94	6	0.6 ± 0.2	148 ± 95

The allosteric model (*5*, *25*, *26*) postulates that nAChRs exist in a conformational equilibrium between a basal (B) state, predominant in the absence of effectors, an active (A) state, intermediate (I) and final (D) desensitized states. The fractional amount of I state is given by the amplitude i_f, and for each given ACh concentration X, by the expression:

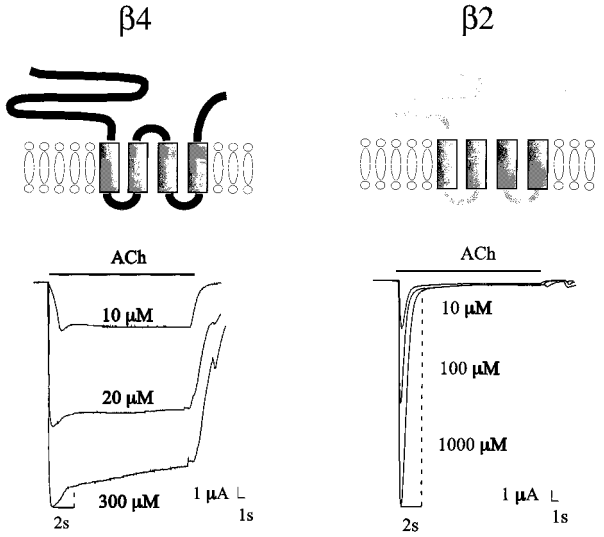

FIGURE 1: Differences in desensitization of α3β2 and α3β4 neuronal nAChRs. Time course of currents evoked by 10, 20, 300 μM ACh on *Xenopus* oocytes expressing the α3β4 receptor (left panel) and by 10, 100, and 1000 μM on *Xenopus* oocytes expressing the α3β2 receptor (right panel). Recordings were performed in control medium (2.5 mM Ca^{2+}) at holding potential of −100 mV. Note that major differences are observed between the time course of desensitization in α3β4 and α3β2 receptors. The biphasic nature of desensitization appears more clearly on traces obtained with α3β4 receptors, and the time for measuring the extent of desensitization approximately matches the break between fast and slow components (2 s after the peak amplitude).

$$\bar{I} = \frac{(1 + X/K_I)^2}{(1 + X/K_I)^2 + {}^{AI}L(1 + {}^{AI}cX/K_I)^2 + {}^{BI}L(1 + {}^{BI}cX/K_I)^2} \qquad (2)$$

where ${}^{AI}c = K_I/K_A$; ${}^{BI}c = K_I/K_B$; K_A, K_B, and K_I are the affinities of the open state A, resting state B, and intermediate desensitized state I, respectively; and ${}^{BA}L = [B]/[A]$, ${}^{AI}L = [A]/[I]$, and ${}^{BI}L = [B]/[I]$ (*28*). The fit of i_f values as a function of X by eq 2 gives plausible values for these parameters.

RESULTS

Desensitization of α3β2 and α3β4 Receptors. The first step in our work was to reinvestigate the functional properties of α3β2 and α3β4 receptors in response to agonist applications. The α3β2 and α3β4 receptors were reconstituted in *Xenopus* oocytes, and ACh was applied at concentrations ranging from 2 to 1000 μM for 15 s. Activation was observed as a rapid current increase within the first 100 ms of recording, whereas desensitization was visualized during the 15 s of recording (Figure 1). Under these experimental conditions, onset of the current was not significantly different between α3β2 and α3β4 receptors. Both receptors displayed robust currents when challenged by a saturating ACh concentration (11 ± 1 μA for α3β2; 15 ± 1.9 μA for α3β4; see Table 1), with EC_{50} values of 65.2 ± 19.8 μM and 16.7 ± 3.2 μM and Hill values of 0.89 ± 0.17 and 1.27 ± 0.22, respectively. The most striking difference was observed in desensitization, thus confirming previously published work (*12*, *27*).

Table 1: Maximal Current Intensities Measured for α3β2, α3β4 Receptors, and Constructs C1−C10 and Desensitization Ratio *D*, as Defined by the Percentage of Currents Remaining after 2 s of Recording, Normalized to the Maximal Amplitude of Currents

	I (nA) ± SEM	*n*	*D* (%) ± SEM	*n*
α3β2	11000 ± 1549	12	94 ± 2	4
α3β4	15147 ± 1914	12	6 ± 2	4
C1	6165 ± 1333	12	78	2
C2	13821 ± 2032	12	16 ± 5	4
C3	343 ± 161	6	57 ± 5	4
C4	2227 ± 869	7	77 ± 4	4
C5	4233 ± 1021	10	72 ± 2	4
C6	1619	2	96	2
C7	6068 ± 804	4	50 ± 7	4
C8	3450 ± 150	3	20 ± 3	3
C9	184 ± 66	7	74 ± 10	3
C10	3319	2	52	2

Desensitization observed during 15 s ACh pulses exhibited a fast and a slow phase (Figure 1). The break between the fast and the slow phase occurred within 1s after reaching the peak of current amplitude; we thus characterized desensitization by measuring the current remaining 2 s after this peak, as compared to the maximal amplitude, yielding a ratio D (see Materials and Methods), which describes the fraction of currents that have been desensitized (Table 1). This ratio D was found to be high (94 ± 2%) for fast desensitizing receptor α3β2, and low for slow desensitizing receptor α3β4 (6 ± 2%, Table 1), in agreement with previous studies (*12*, *15*).

The N-Terminal, Extracellular Domain of the β-Subunit Determines the Differences in Desensitization Kinetics of α3β2 and α3β4 Receptors. Chimeras constructed between the β2 and the β4 subunits were produced to assess which part of the molecule was responsible for fast desensitization (Figure 2, panel A). Early biochemical experiments, performed on the muscle-type nAChR (*28*) have shown that each subunit adopts a common transmembrane topology including a large, N-terminal domain, that carries the ACh binding pocket, and four transmembrane domains with a large cytoplasmic loop between the third and fourth transmembrane segments (Figure 1). The first chimera (construct C1) was designed by fusing the N-terminal domain of the β2 subunit (residues 1−212 referenced to the β4 numbering system, Figure 2, panel B) and the C-terminal domain of the β4 subunit, joined at the level of a restriction site within the first transmembrane segment (the *Bbs*I site at L233). For the reverse construct, C2, the N-terminal domain of the β4 subunit and the C-terminal domain of the β2 subunit were fixed at the corresponding position. Current traces evoked by saturating ACh concentrations are presented in Figure 3, panel A, normalized to their maximal value: they clearly show a fast desensitization at 2 s for receptors containing C1 ($D = 78\%$, Table 1) and less desensitization at 2 s for those obtained with C2 ($D = 16 \pm 5\%$). In contrast, little increase in the fast desensitization amplitude was measured between α3β4 ($D = 6 \pm 2\%$) and C2 (16 ± 5%) and a small decrease between α3β2 ($D = 94 \pm 2\%$) and C1 ($D = 78 \pm 2\%$). These results therefore emphasize the crucial involvement of the N-terminal domain of the β-subunit in the desensitization of α3β2 and α3β4 receptors, when measured as a phenotypic fractional current remaining after the fast component of current decline. A minor role is assigned to the C-terminal half, containing transmembrane domains and cytoplasmic loop.

Both Halves of the N-Terminal Domain of β2 Confer Fast Desensitization upon α3β4 Receptors. To determine which portions of the N-terminal domain of the β-subunit were crucial in determining fast desensitization, additional chimeric constructs were produced. Either residues 1−95 were exchanged between β2 and the β4 subunits using a *Bsr*GI restriction site (Figure 2, panel B), thus yielding construct C3, or residues 96−212 of the β2 subunit were substituted into the β4 sequence using the same *Bsr*GI and *Bbs*I sites as used for constructs C1 and C2, thus yielding construct C4. Both constructs C3 and C4 were expressed with the α3 subunit and challenged by a saturating ACh concentration (1000 μM). Figure 3, panel B illustrates the relative contribution of residues 1−95 and 96−212, and reveals that both sequence exchanges cause a significant increase in desensitization, considerably augmenting the *D* ratio. Construct C3 produced a 10-fold increase ($D = 57 \pm 5\%$) and construct C4 produced a 13-fold increase ($D = 77 \pm 4\%$) as compared to the α3β4 receptor ($D = 6 \pm 2\%$).

For the reciprocal experiments, slow desensitization at 2 s was not conferred on α3β2 by either portion 1−95 or 96−212 of the β4 sequence. Receptors containing chimeric subunits C5 and C6 (Figure 3, panel C) indeed still exhibited fast desensitization kinetics (C5, $D = 72 \pm 2\%$; C6, $D = 96\%$), illustrating that slow desensitization is not solely determined by the first or the second half of the N-terminal domain of the β4 subunit.

Thus, fast desensitization was readily conferred upon α3β4 receptors by either half of the N-terminal domain of the β2 subunit in a distinct manner, whereas slow desensitization of α3β4 receptors required the entire N-terminal domain of the β4 subunit. According to our results, slow desensitization of β4-containing receptors, measured 2 s after the maximum of response is reached, appears as a particular phenotype, easily disrupted by separate portions of the β2 sequence. In contrast, the fast desensitization of α3β2 receptors can be considered as a default phenotype occurring if one or the other half of the N-terminal domain of the β4 subunit is exchanged.

Residues 1−42 and 72−89 from the β2 Sequence Individually Confer Fast Desensitization on α3β4 Receptors. The first half of the N-terminal domain of the β subunit was divided into two regions: residues 1−42 on one hand, and residues 43−95 on the other hand. Transfer of residues 1−42 from the β2 subunit into the β4 subunit yielded chimera C7 (Figure 4, panel A), whereas for residues 43−95, the dissection was directly targeted to a particularly nonconserved stretch of 18 residues between β2 and β4 sequence (positions 72−89, see Figure 4). In this last operation, transfer of residues 72−89 was carried out in two steps: first, by introducing a restricted cassette of five residues (77−81), yielding construct C8, and second, by adding residues 72−76 and 82−89 at both ends, thus producing chimera C9.

When expressed with the α3 subunit, construct C7 displayed robust currents (see Table 1) that desensitized rapidly (Figure 4, panel A). A nearly 8-fold increase was consistently measured in the *D* ratio of desensitization as compared to the α3β4 receptor (α3β4: $D = 6 \pm 2\%$; C7: $D = 50 \pm 7\%$; Table 1). This ratio was almost as high as for receptors containing construct C3 ($D = 57 \pm 5\%$), in

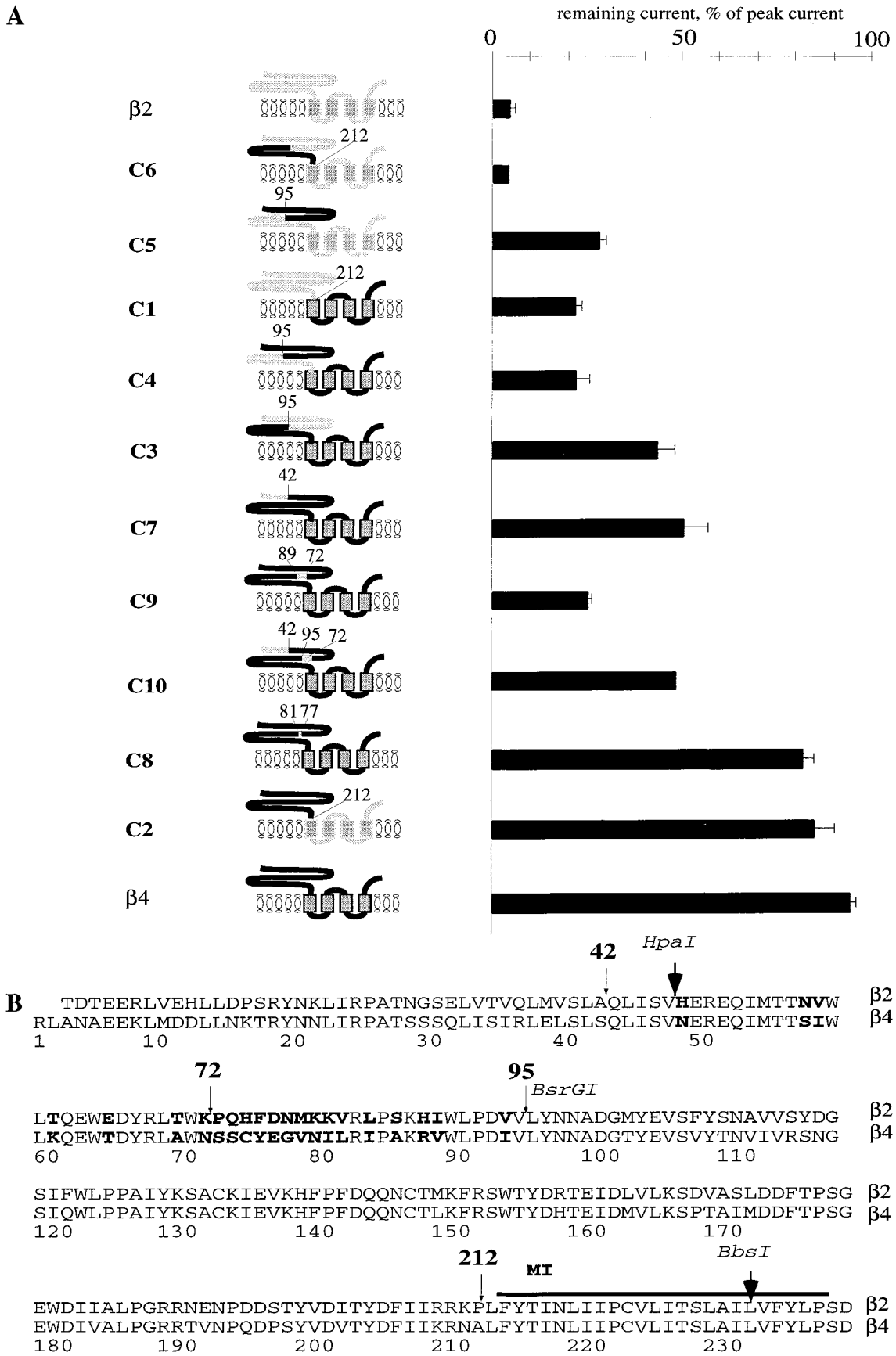

FIGURE 2: Chimeras constructed with the $\beta 2$ and the $\beta 4$ subunits. (A) Schematic representation of the chimeras and fraction of remaining currents measured 2 s after the peak response (see Materials and Methods). Each data point represents the average value of 2−5 cells. (B) Points of chimerization that served for the construction of chimeras C1−C10. Key positions of amino acids 42, 72, 77, 81, 89, 95, and 212 are indicated. Restriction sites were *Bbs*I at position 212 (naturally contained in the sequence), *Bsr*GI at position 95, and *Hpa*I (introduced by mutagenesis) at position 46; microchimeras were specifically designed by site-directed mutagenesis using appropriate oligonucleotides in the region 72−89.

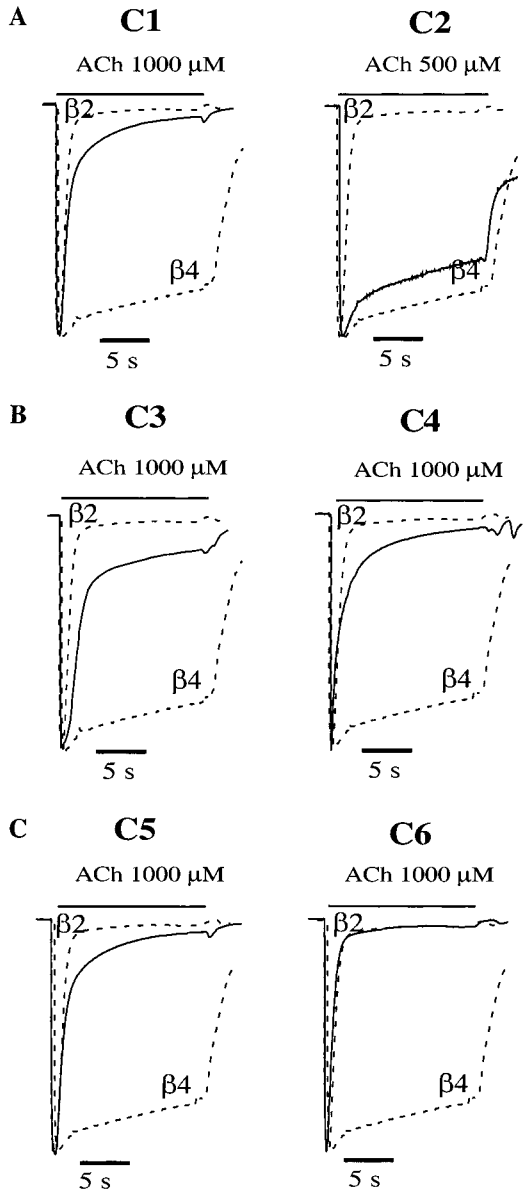

FIGURE 3: Role of the N-terminal domain of the β-subunit in the rate of desensitization of $\alpha3\beta2$ and $\alpha3\beta4$ receptors. Panels A, B, and C. Time course of currents evoked by saturating concentrations of ACh (1000 μM; 15 s) on C1−C6 chimeras expressed in combination with the $\alpha3$ subunit (thick lines), as compared to the wild-type receptors $\alpha3\beta2$ and $\alpha3\beta4$ (dashed lines). For comparison, all currents were normalized to unity.

which the entire stretch 1−95 of the $\beta2$ subunit was present. This finding suggested that key elements may be expected to lie between residues 1−42, while residues 43−95 should be less important. However, by examining constructs C8 and C9, we found that other important modulators of desensitization were also present within these positions.

Chimera C8, producing robust currents (3450 ± 150 nA, Table 1), showed a small acceleration of desensitization as compared to the original sequence of the $\beta4$ subunit ($\alpha3\beta4$: $D = 6 \pm 2\%$; C8: $D = 20 \pm 3\%$). This resulted in a visible, although modest peak of fast desensitization as seen on the traces of Figure 4. In contrast, receptors reconstituted with chimera C9 displayed small but analyzable currents (184 ± 66 nA, Table 1) that revealed an additional, 3-fold, increase in their fast component of desensitization, with a large effect on current decline (see Figure 4, panel B). The overall stretch of residues thus resulted in a final ratio $D = 74 \pm 10\%$, which was the most striking effect noted for a restricted sequence on the kinetics of fast desensitization in our study. The total effect of residues 72−89 was a 12-fold increase in D ratio between $\alpha3\beta4$ and C9, and significantly exceeded the effect of the entire stretch 1−95 (construct C9: $D = 74 \pm 10\%$; construct C3: $D = 57 \pm 5\%$). According to our results, the relative roles of residues 1−42 and 72−89 may be viewed as putatively linked, and possibly engaged in complex intramolecular interactions. Slow desensitization at 2 s of $\alpha3\beta4$ receptors thus appears as a vulnerable property that can be interrupted by restricted changes in the amino acid sequence of the $\beta4$ subunit.

Effects of Portions 1−42 and 72−89 from the β2 Subunit on Fast Desensitization are Nonadditive. Portions 1−42 (C7) and 72−89 (C9) from the $\beta2$ subunit cause significant decrease in the level of currents measured 2 s after the peak response ($D = 50$ and 74%, respectively). Their combined effect was examined by constructing a tenth chimera, C10, for which residues 48−70 from the $\beta4$ sequence were introduced into construct C3 (residues 43−47 and 71 are conserved residues between $\beta2$ and $\beta4$ sequences). The resulting chimera thus contained both segments separated by a stretch of 30 amino acids (see Figure 2, panel A). Receptors reconstituted with chimera C10 were functional and displayed robust currents (3319 nA, Table 1); even though a marked component of fast desensitization persisted (Figure 4, panel C), the currents did not desensitize more rapidly than those generated by the construct C9. The D ratio (52%, see Table 1) was intermediate between those of C7 (50 ± 7%) and C9 (74 ± 1%), although closer to the C7 phenotype. This showed that the effects of portions 1−42 and 72−89 were not additive and that the action of these portions could not be considered independently from each other in the N-terminal of the $\beta2$ subunit.

Desensitization Levels after 25-min ACh Exposure. The effects of prolonged ligand exposure on the state of activity of the $\alpha3\beta2$ and $\alpha3\beta4$ receptors and combined constructs C1−C5 were examined following a standard 25 min ACh application. Various concentrations of ACh were applied over this time period, and currents were allowed to stabilize up to a certain extent, while not exceeding 25 min of recording so as to allow several measurements to be made on the same cell (see Figure 5, panels A and B). The fraction of remaining activatable receptors was estimated by a brief ACh pulse (2 s) at a concentration close to the EC_{50} (*29*). This fraction decreased when the ACh exposure concentration was increased, indicating that a higher fraction of the remaining receptors was desensitized. The reduction in the fraction of activatable receptors is shown in inhibition curves of both $\alpha3\beta4$ and $\alpha3\beta2$ receptors (Figure 5, panels A and B) and characterized by the half-maximal concentration of ACh necessary to cause a 50% inhibition of the test response (IC_{50}).

In this 25-min application protocol, the inhibition curve of $\alpha3\beta2$ receptors was shifted to the left as compared to the activation curve (75-fold difference between these values: $EC_{50} = 29 \pm 6$ μM and $IC_{50} = 0.4 \pm 0.01$ μM, mean of 4 cells). For $\alpha3\beta4$ receptors, the IC_{50} value remained close to the EC_{50} (10 ± 2.6 μM and 14 ± 2.9 μM, respectively, mean of 4 cells). Thus, $\beta2$ and $\beta4$ subunits are found to make major contributions to the sensitivity of the receptors to prolonged

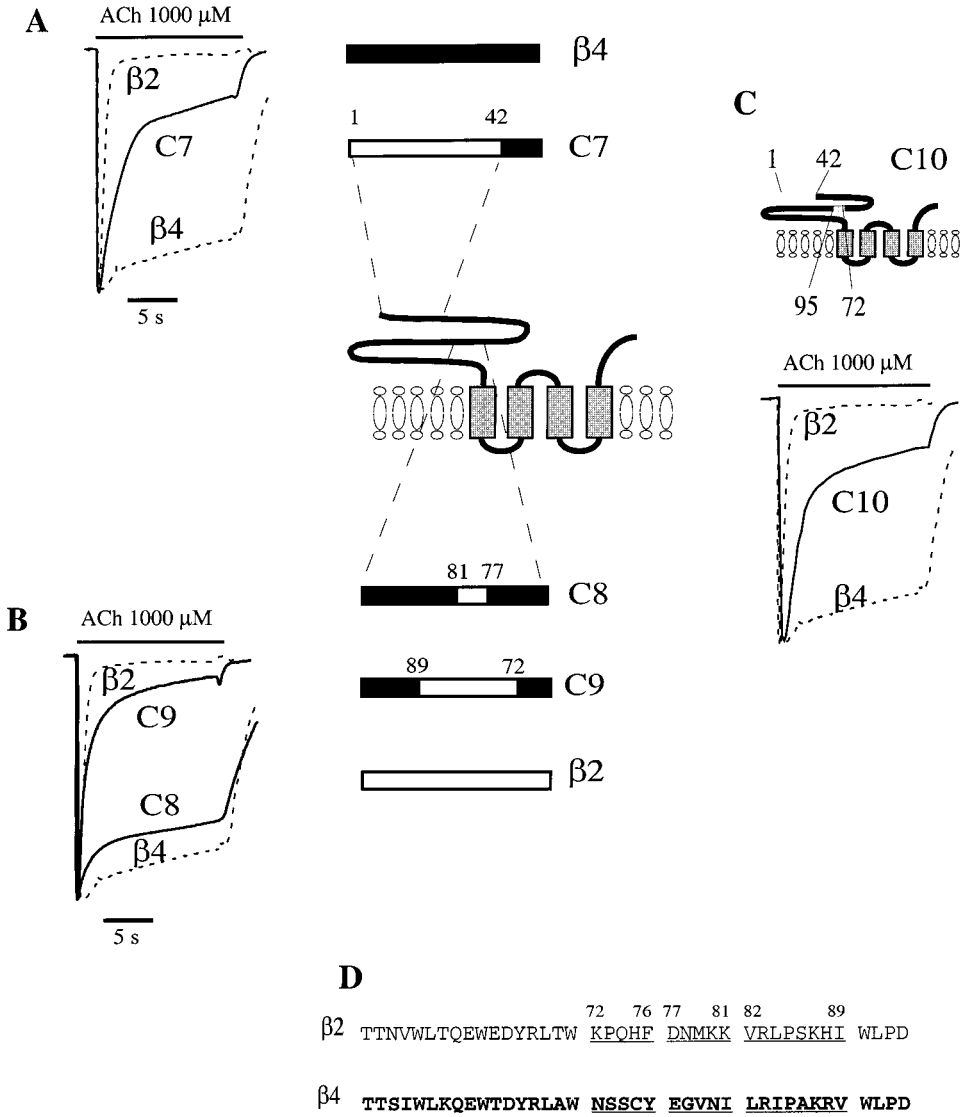

FIGURE 4: Role of residues 1−42 and 72−89 in contributing to fast desensitization. (A) Residues 1−42 were transferred from β2 into the β4 sequence, using a mutagenesis-created *Hpa*I restriction site at position 46, and yielding construct C7. (B) Residues 77−81 were transferred from the β2 into the β4 sequence, yielding construct C8, while transfer of residues 72−89 were transferred from the β2 into β4 sequence, yielding construct C9. (C) Oocytes expressing the α3 subunit with one of the constructs C7−C10 were challenged by a saturating ACh concentration (1000 μM; 15 s). Currents were normalized and represented as in Figure 3. Combination of residues 1−42 and 72−89 from the β2 sequence both inserted into the β4 sequence yielded chimera C10. (D) Alignment of the amino acid sequences of the β2 and β4 subunits in the N-terminal region 56−93.

ligand exposure. This effect was dissected by examining constructs C1−C5, with results presented in Figure 5.

Separation between inhibition and activation curves was still present in receptors containing the chimeric subunit C1 (residues 1−212 of the β2 sequence) but not in receptors reconstituted with the reverse construct, C2 (residues 1−212 from the β4 sequence). This indicates that the N-terminal domain is determinant for the sensitivity to the long-term ligand exposure. Progressing in the molecular dissection, it appeared that both portions 1−95 or 96−212 of the β2 sequence were capable of creating a substantial difference between the activation and the desensitization curves typical of the α3β2 receptor (30-fold difference, constructs C3 and C4). In contrast, the entire N-terminal domain (residues 1−212) of the β4 subunit was required to maintain desensitization and activation within the same concentration window. These results reveal the low sensitivity of α3β4 receptors to prolonged ligand exposure, as well as their slow desensitization kinetics at 2 s, as constrained properties requiring the simultaneous presence of several residues located in different parts of the N-terminal domain. High sensitivity and fast desensitization kinetics, in contrast, appear as a default property of α3-containing receptors.

DISCUSSION

Desensitization is a widespread phenomenon among ligand-gated ion channels (*8*). In some cases, its particularly slow kinetics led to their characterization as "nondesensitizing receptors" (*30*, *31*). A way of addressing the problem of structural elements underlying desensitization is to confer fast desensitization upon such receptors. We have chosen the typical, well described couple of α3β2 and α3β4 nAChRs to perform such a kinetic transfer, based on previous studies comparing their desensitization features (*12*). This approach permitted the localization of determinant protein segments in the β-subunit, by examining residues that were not conserved between the β2 and the β4 subunit (*10*, *22*, *32*).

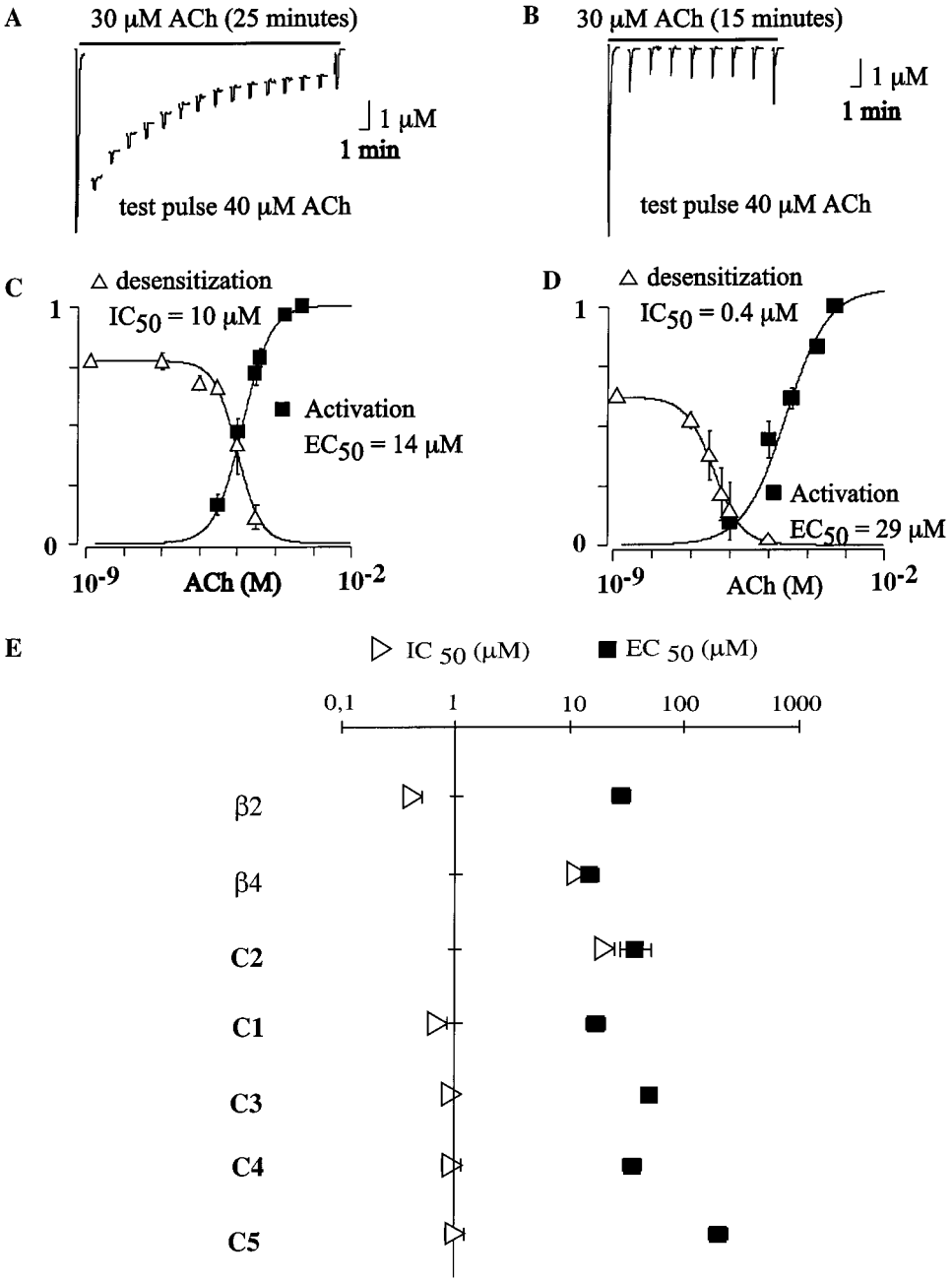

FIGURE 5: Sensitivity to long-term ACh exposure of receptors containing constructs C1−C5. (A) Effects of a prolonged exposure to a fixed ACh concentration (30 μM) at $\alpha3\beta4$ receptors (here, 30 μM) were measured by continuously superfusing the desensitizing concentration. Periodic applications of brief ACh test pulses (40 μM, 2 s) allowed monitoring of the amount of activatable receptors. (B) Stabilization is more rapid for $\alpha3\beta2$ receptors. The slight differences observed in the amplitude of the test pulse response, particularly for the last test, correspond to variability of the oocyte measurement. Desensitization is significant, but longer recordings were complicated by instability of the baseline. (C and D) activation and desensitization curves presented on the same graph for $\alpha3\beta4$ and $\alpha3\beta2$ receptors. Note the major separation between both curves in the case of $\alpha3\beta2$ receptors, respectively. (E) EC_{50} values (filled squares) and IC_{50} values (open triangles) of receptors obtained by expression of the $\alpha3$ subunit with the chimeras C1−C5. Each data point represents the average value of 2−5 cells. Error bars correspond to the SEM.

Desensitization Kinetics of nAChRs: Crucial Involvement of the N-Terminal Domain of the β-Subunit. Calculating the ratio $D = (I_m - I_{2s})/I_m$ as defined in Materials and Methods, the desensitization of $\alpha3\beta2$ receptors (94% of total currents) and $\alpha3\beta4$ receptors (6%) were in marked contrast, thus confirming previous observations (*12*, *15*). We found that the N-terminal portion of the $\beta2$ subunit was involved in accelerating desensitization kinetics (residues 1−212), while the same portion of the $\beta4$ subunit caused a decrease in desensitization. This suggested that desensitization could be driven by autonomous reorganizations taking place within the extracellular domain of the receptor, relatively independently from intracellular interactions mediated by the cytoplasmic loop. Because we have investigated only the N-terminal domain, changes in receptor phosphorylation, a phenomenon known to influence desensitization through action on residues within the cytoplasmic loop, could not be addressed in our study. Moreover, phosphorylation differences may also occur between *Xenopus* oocytes and neurons. The segments of the N-terminal domain that were involved in these interactions were further analyzed by a strategy of microchimeras.

The limits of this approach were revealed by chimeras C3, C4, C5, and C6, that all displayed fast desensitizing kinetics. This strategy did not allow identification of unique elements governing desensitization in the N-terminal domain but rather suggested that such elements were diverse. Still, the analysis of nonconserved residues in the portion 1−95 allowed us to identify a few residues (among others) that could specifically transfer fast, but not slow desensitization at 2 s.

In most cases, the mutations were not "additive", i.e., the effect of a given substitution was dependent on the amino acids present in other regions. For example, residues 96−212 almost fully account for the fast desensitization caused by residues 1−212 of the β2 sequence (see constructs C4 and C1), whereas residues 1−95 yielded only a 57% desensitization ratio *D*. Also, combining mutations 72−89 and 1−42 did not yield stronger desensitization than residues 72−89 alone, but rather significantly less (48 versus 26%), whereas 1−42 produced a 50% desensitization ratio *D*. In the direction of transferring β4 segments into the β2 subunit, the combined effect of portions 1−95 and 96−212 was also far above the desensitization that would be expected if both segments were acting independently (C5: $D = 72\%$; C6: $D = 96\%$ and C2: $D = 16\%$). All these evidences point to multiple interactions within the N-terminal domain of the β-subunits during desensitization.

The nature of these interactions remains, however, to be elucidated. Yet, the finding that the entire N-terminal extracellular domain accounts for the desensitization kinetics fits the idea that this domain constitutes a structural unit which folds autonomously (*36*, *37*). A tentative 2D representation of a typical nicotinic subunit, which incorporates secondary structure predictions and the biochemical data collected so far about the ACh binding site at the subunit interface (*38*), suggests that the 67−212 domain is folded into an all β core, with residues 67−79 placed at the top of the fold (to fit data from electron microscopy; ref *37*). Accordingly, a folding axis is proposed for the 1−65 region and may mediate the interaction between segments 1−42 and 72−89 inferred from our experiments.

It is noteworthy that the major differences in the desensitization of $\alpha3\beta2$ and $\alpha3\beta4$ receptors result from differences in the amplitude of the fast component of desensitization (see Materials and Methods) rather than from differences in the time constants. The break between the fast and the slow components observed during current decay is assumed to correspond to the transitory stabilization of an intermediate desensitized state I (*3*, *4*). The relative amplitude of this plateau at saturating concentrations of ACh thus represents the relative thermodynamic stability of the biliganded I state as compared to the basal activatable B, active A (with open channel) and desensitized D states (see Materials and Methods and ref *26*). According to the simulations based on these assumptions (see eq 2 in Materials and Methods, data not shown), differences between wild-type $\alpha3\beta2$ and $\alpha3\beta4$ receptors involve both differences in the affinity ratio constant ^{AI}c and in the isomerization constant ^{AI}L. The C9 chimera (residues 72−89 from the β2 sequence transferred into the β4 subunit) would, in contrast, possess the affinity ratio of the $\alpha3\beta4$ receptor and the isomerization constant ^{AI}L of the $\alpha3\beta2$ receptor. This is consistent with the notion that residues 72−89 do not act directly at the level of the binding pocket but possibly stabilize the folded structure of a distant region of the receptors that would be more directly involved in desensitization. Such other regions might ultimately be the pore-lining residues at which mutations dramatically alter desensitization kinetics (*40*), but these residues could not be implicated in our study since they are conserved between the β2 and β4 subunits.

Our overall finding is the multiplicity of N-terminal residues conferring desensitization when replaced in the β4 sequence. This complements the results of previous studies where residues involved in desensitization kinetics of neuronal nAChRs had been identified mainly in the transmembrane regions, particularly in the channel domain [e.g., mutation L247T or L247N (*40*)]. N-terminal residues were identified by transferring near-binding-site cassettes from the α4 subunit to the α7 subunit (*29*), but only the steady-state levels of desensitization were then altered. Our chimeras designed in the N-terminal domain thus allow us to better delineate the sequences underlying short-term kinetics and to propose that nonconserved residues within the N-terminal domain of heteromeric receptors may act together with the readily identified amino acids of transmembrane regions.

The multiplicity of elements individually conferring desensitization underlines the fact that desensitization is a strong property of α3-containing receptors. The identified portions of the N-terminal domain, indeed, played asymmetric roles depending on their sequence origin: when taken from the β2 sequence, residues 1−42, 72−89, and 96−212 separately transferred fast desensitization at 2 s (constructs C1, C3, C4, C7, or C9); when taken from the β4 sequence, individual regions failed to produce extensive slow desensitization at 2 s, except when the entire N-terminal domain of the β4 subunit was transferred. N-terminal elements were thus individually efficient in the strategy to confer desensitization upon nondesensitizing receptors but not in abolishing desensitization of $\alpha3\beta2$ receptors.

Prolonged Desensitization of nAChRs: Similar Requirements as for Short-Term Kinetics. The effects of prolonged ACh exposures were examined on both $\alpha3\beta2$ and $\alpha3\beta4$ receptors and five other chimeras. A time period of 25 min was chosen, to reach relative steady-state levels of currents (clearly reached for $\alpha3\beta2$, and putatively for $\alpha3\beta4$ receptors) and to adjust the repeated ACh applications on the same cell to the survival time of oocytes. Although full equilibrium may not be reached in certain cases (*25*), this protocol should nevertheless mimic to a large extent the action of ACh under physiological conditions. Our finding that two different portions (residues 1−95 and 96−212) of the β2 sequence can separately increase the sensitivity to long-lasting applications of ACh upon $\alpha3\beta4$ receptors strikingly parallels the effects of both these portions on fast desensitization kinetics. Our data on the crucial role played by residues 1−212 of the β-subunit can be linked to the recent finding (*41*) that the N-terminal domain of the α4 versus the α3 subunit carries elements involved in the inactivation of $\alpha4\beta2$ receptors by prolonged applications of nicotine. Both α- and β-subunits therefore play a role in the long-term effects of cholinergic ligands.

CONCLUSION

Desensitization has been shown to contribute to synaptic plasticity through its action on glutamate (*8*, *42*), GABA A

(*43*), and AMPA receptors (*44*). Desensitization of neuronal nicotinic receptors is mainly involved in the modulation of neurotransmitter release underlying in nicotine dependence (*45*), putatively occurring through the action of presynaptically located receptors (*46*). In this respect, the time course of acetylcholine-evoked depolarization and in turn, calcium entry (*47*, *48*), the modulation of dopamine, norepinephrine, or glutamate release (*49*, *50*) may show dependence on desensitization kinetics. Our finding that several nonoverlapping stretches of residues from the β2 subunit can individually confer desensitization complements the known contribution of channel-lining residues (*40*) and N-terminal domains carried by the α-subunit (*29*) to desensitization. The findings also suggest that desensitization is a strong and fundamental property of α3 channels and that the slow desensitization observed shortly after the peak of response requires the simultaneous presence of several residues disseminated along the N-terminal domain of the β4 subunit. The mutations identified herein may help investigating the suspected relationships between desensitization and upregulation (*24*), the well-known process potentially underlying nicotine dependence (*32*, *51*).

ACKNOWLEDGMENT

We thank Jim Boulter for providing cDNA clones from the β2 and β4 rat gene of nicotinic subunits and Anne Devillers-Thiéry, Régis Grailhe, and Yoav Paas for critical reading of the manuscript and valuable discussions.

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BI0020022