

First and Second Transmembrane Segments of $\alpha 3$, $\alpha 4$, $\beta 2$, and $\beta 4$ Nicotinic Acetylcholine Receptor Subunits Influence the Efficacy and Potency of Nicotine

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ABSTRACT

The first three transmembrane segments (M1–M3) of human nicotinic acetylcholine receptors (nAChRs) have been implicated in determining the efficacy of nicotine by studies of $\alpha 3/\alpha 4$ subunit chimeras (Kuryatov et al., 2000a). Nicotine has full efficacy on the $\alpha 4\beta 2$ nAChR and partial efficacy on the $\alpha 3\beta 2$ nAChR. Now, we have exchanged individually three amino acids between the $\alpha 4$ and the $\alpha 3$ subunits at positions 226(M1), 258(M2), and 262(M2). Also, similar exchanges were made in the $\beta 2$ and $\beta 4$ subunits at positions 224(M1), 226(M1), and 254(M2) (using α subunit numbering). Expression of these mutated nAChRs in *Xenopus laevis* oocytes showed that the mutated M1 amino acids were important in influencing the potency

of ACh and nicotine. It is hypothesized that these M1 amino acids affect the stability between the resting and activated states of the nAChR. M2 amino acids altered the efficacy of nicotine, usually without altering its potency. When the residue located at position 258 in the M2 region of the α subunit was valine (as in the $\alpha 3$ subunit), the resulting nAChR exhibited partial efficacy for nicotine that was voltage-dependent. Therefore, we believe that these M2 amino acids contribute to the formation of a binding site for nicotine in the $\alpha 3\beta 2$ nAChR channel, which results in a low-affinity channel block, causing the lower efficacy of nicotine on this nAChR.

Nicotinic acetylcholine receptors (nAChRs) are formed from five homologous subunits organized around a central cation channel (Lindstrom, 2000; Grutter and Changeux, 2001; Karlin, 2002). Each subunit is composed successively of a large N-terminal extracellular domain, three transmembrane segments (M1–M3), a large cytoplasmic domain, and another transmembrane segment (M4) that ends in a small extracellular C-terminal domain. The channel lining is formed by M2 and the top third of M1 from each subunit (Wilson and Karlin, 2001).

Seventeen nAChR subunits have been cloned ($\alpha 1$ – $\alpha 10$, $\beta 1$ – $\beta 4$, γ , δ , and ϵ) (Lindstrom, 2000; Grutter and Changeux, 2001; Karlin, 2002). These subunits can combine to form a variety of nAChR subtypes, including homopentamers [e.g., ($\alpha 7$)₅], heteromeric arrangements of nAChRs with two kinds of subunits [e.g., an ($\alpha 3$)₂($\beta 4$)₃ of autonomic ganglia or an ($\alpha 4$)₂($\beta 2$)₃ nAChR of brain], or more complex heteromers [e.g., an ($\alpha 1$)₂ $\beta 1\delta\epsilon$ nAChR of skeletal muscle]. In simple heteromeric neuronal nAChRs, two ACh binding sites form at the interfaces of an α and β subunit in the extracellular domain close to the N terminus of M1 (Karlin, 2002). Binding of agonists at these sites causes transient opening of the

channel gate, which is believed to be near the cytoplasmic end of the channel between M1 and M2 (Wilson and Karlin, 2001). The transition from the closed to open state is thought to involve conformational changes in all five subunits.

Nicotine, a potent agonist, is the addictive component of tobacco (Dani et al., 2001). Whereas nAChRs may typically be exposed to ACh for milliseconds, tobacco users are exposed to nicotine for many hours at sustained serum nicotine concentrations ranging from 0.2 μ M to transient peak levels of near 1 μ M after inhaling smoke. Such agonists as ACh and nicotine initially activate nAChRs, then desensitize them, and upon prolonged exposure can cause an increase in the amount of nAChRs. The extent of these different effects is dependent on the nAChR subunit composition and may also depend on the cell type in which the nAChRs are expressed (Olale et al., 1997; Wang et al., 1998; Nelson et al., 2001).

Nicotine is a partial agonist on human $\alpha 3\beta 2$ nAChRs, but it exhibits greater efficacy on human $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs (Wang et al., 1996; Olale et al., 1997; Gerzanich et al., 1998). Therefore, both subunits, α and β , seem to contribute to the efficacy of nicotine. $\beta 2$ subunits are associated with higher ligand-binding affinity and more rapid desensitization than $\beta 4$ subunits (Fenster et al., 1997; Parker et al., 1998; Bohler et al., 2000). One mechanism that could account for the partial agonist activity of nicotine is that its binding at the

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agonist-binding site does not efficiently trigger the conformational change to the open channel state. Another possible mechanism is that nicotine efficiently triggers channel opening but then binds weakly to a site in the channel, so that it blocks or slows down the passage of cations through the channel. Studies with chimeric nAChRs suggested, but did not prove, that nicotine is a partial agonist on $\alpha 3\beta 2$ nAChRs because it blocks the channel (Kuryatov et al., 2000a). A chimera consisting of the extracellular domain of $\alpha 3$ with the remainder of $\alpha 4$, when coexpressed with $\beta 2$, formed normal $\alpha 3\beta 2$ -like ACh binding sites but had an $\alpha 4\beta 2$ -like channel. Nicotine was fully efficacious on this nAChR. Conversely, a chimera consisting of the extracellular domain of $\alpha 4$ joined to the remainder of $\alpha 3$, when coexpressed with $\beta 2$, formed normal $\alpha 4\beta 2$ -like ACh binding sites but had an $\alpha 3\beta 2$ -like channel. Nicotine was only partially efficacious on this nAChR (Kuryatov et al., 2000a). Here, we report single and double amino acid chimeras between the $\alpha 3$ and $\alpha 4$ subunits or the $\beta 2$ and $\beta 4$ subunits, which precisely map amino acids in the channel-lining M2 transmembrane domain of these subunits responsible for the low efficacy of nicotine. Also, in M1, we find amino acids that can influence the potency of ACh and nicotine on nAChRs without altering efficacy.

Materials and Methods

Subunit DNA Clones. The cDNA for the human $\alpha 4$ subunit was cloned into a pSP64 (polyA) vector (Promega, Madison, WI) (Kuryatov et al., 1997). The $\alpha 3$ subunit was cloned into a pcDNA I vector (Invitrogen, Carlsbad, CA) (Wang et al., 1996) and subcloned into *Hind*III and *Bam*HI sites of a pSP64 vector. The $\beta 2$ subunit was cloned into a pSP64 vector (Anand and Lindstrom, 1990). The $\beta 4$ subunit was cloned into a pcDNA I vector (Invitrogen) (Gerzanich et al., 1997). These vectors were used for all mutations that were produced and for subsequent RNA production.

Production of Mutants. All point mutations were made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Sense and antisense oligonucleotide primers that contained the desired mutation were produced (Invitrogen). All primers used had a melting temperature greater than 76°C. The oligonucleotide primers (125 ng each) were annealed (55°C) to the template DNA (50 ng), and the primers were extended (68°C) by using *Pfu* Turbo DNA polymerase (Stratagene). Then, the template DNA was

degraded with *Dpn*I, an endonuclease that recognizes methylated and hemimethylated DNA. The mutant DNA was then transformed into Epicurian Coli XL1-Blue supercompetent cells (Stratagene). All products were subsequently sequenced in the region of the mutation to ensure the accuracy of this procedure. cRNA corresponding to each mutation was produced using the mMessage mMachine (Ambion, Austin, TX). The oligonucleotide primer sequences used to produce mutants are shown in Table 1.

Expression of Clones and Mutants. Oocytes were obtained from *X. laevis* (Xenopus I, Ann Arbor, MI). The oocytes were surgically removed and placed in L-15 medium [50% Leibovitz's L-15 medium (Invitrogen), 10 mM HEPES, pH adjusted to 7.5 with NaOH, containing 50 U/ml of penicillin, 50 ug/ml of streptomycin, and 50 ug/ml of gentamicin]. Oocytes were rinsed in calcium-free OR2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES, pH adjusted to 7.5 with NaOH) then defolliculated in this buffer containing 2 mg/ml of collagenase A (Sigma-Aldrich, St. Louis, MO) for approximately 2 h.

Stage V–VI oocytes were selected and injected with 5 to 15 ng of cRNA for each of the α and β subunits in a total volume of between 10 and 23 nl. After the injection, the oocytes were maintained at semisterile conditions at 18°C in diluted L-15 media.

Electrophysiological Recordings. A standard two-microelectrode voltage clamp amplifier (Oocyte Clamp OC-725; Warner Instrument Corp., Hamden, CT) was used to measure the currents generated in the oocytes in response to the application of an agonist, either nicotine, cytosine, or ACh. Electrophysiological recordings were performed on days 3 through 8 after cRNA injection as described previously (Gerzanich et al., 1995). The borosilicate electrodes were filled with 3 M KCl and had a typical resistance of between 0.5 and 2 M Ω . All recordings were digitized using Mac Lab software and hardware (ADInstruments Pty Ltd., Castle Hill, Australia) and stored on an Apple Macintosh computer. Data were analyzed using KaleidaGraph (Abelbeck/Synergy, Reading, PA) and fitted using a modified Hill equation to determine the Hill coefficient and EC₅₀ value: $I_{\text{peak}} = I_{\text{max}} / [1 + (\text{EC}_{50} / [\text{A}]^n)^H]$.

The recording chamber was continually perfused with ND-96, a physiological saline solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.5, and 0.5 μ M atropine to block muscarinic nAChRs. Application of the agonist was performed using a set of glass tubes. For each subunit combination tested, an initial concentration-response curve was produced to determine the EC₅₀ and EC₁₀₀ values for ACh. Only oocytes that had maximum responses of between 1 and 10 μ A were used for the production of concentration-response curves. Concentration-re-

TABLE 1
Oligonucleotide primer sequences used to produce mutants

$\alpha 4$ (C226F)	
S	5' GCC TGC TCA TCT CCT TCC TCA CCG TGC 3'
AS	5' GCA CGG TGA GGA AGG AGA TGA GCA GGC 3'
$\alpha 4$ (L258V)	
S	5' CTT CCT GCT GGT GAT CAC CGA GAT CAT CCC 3'
AS	5' TCT CGG TGA TCA CCA GCA GGA AGA CGG 3'
$\alpha 4$ (I262T)	
S	5' CAT CAC CGA GAC CAT CCC GTC C 3'
AS	5' GGA CGG GAT GGT CTC GGT GAT G 3'
$\beta 2$ (I224T)(S226L)	
S	5' CCT GTG TGC TCA CCA CCT TAC TAG CCA TCC 3'
AS	5' GGATGG CTA GTA AGG TGG TGA GCA CAC AGG 3'
$\beta 2$ (V254F)	
S	5' GGC GCT CAC GTT CTT CCT GCT GC 3'
AS	5' AGC AGG AAG AAC GTG AGC GCC 3'
$\alpha 3$ (V258L)	
S	5' CGG TGT TTC TCC TGC TCA TCA CTG AGA CC 3'
AS	5' TCT CAG TGA TGA GCA GGA GAA ACA CCG 3'
$\alpha 3$ (T262I)	
S	5' GAT CAC TGA GAT CAT CCC TTC CAC C 3'
AS	5' TGG AAG GGA TGA TCT CAG TGA TCA CC 3'

S, sense; AS, antisense.

sponse curves were produced from two to six oocytes expressing each type of nAChR (see Table 2). These agonist solutions were applied to the recording chamber by manually opening clip valves for 2 to 4 s using a gravity flow setup. Unless stated otherwise, all recordings were performed at a holding potential of -50 mV. Washes of 3 to 5 min were performed between agonist applications to allow full recovery of the maximal response. For measurement of the voltage sensitivity of nicotine efficacy, a single, barely saturating concentration of either ACh or nicotine was applied at each holding potential, with intra-application intervals of 3 min. The series of measurements was always performed in the order -40 through -100 mV. Nicotine efficacy for these experiments was determined by comparing the response to nicotine for that oocyte to the response measured for ACh at each holding potential.

Results

Sequence Differences between $\alpha 3$ and $\alpha 4$ or $\beta 2$ and $\beta 4$ Subunits. Chimeras between the $\alpha 3$ and $\alpha 4$ subunits have shown that, when coexpressed with the $\beta 2$ subunit, amino acids within M1–M3 of the α subunit can determine the efficacy of the response of an nAChR to nicotine (Kuryatov et al., 2000a). Sequence comparison (Fig. 1) shows that there are five amino acid differences between the $\alpha 3$ and $\alpha 4$ subunits in the M1–M3 region. Thus, one or more of these residues of the α subunit should account for the different efficacies of nicotine on the $\alpha 4\beta 2$ and $\alpha 3\beta 2$ nAChRs. Both subunits, α and β , play an important role in determining the ability of nicotine to activate nAChRs (Figl et al., 1992; Hussy et al., 1994). The $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs also differ slightly in the efficacy of their activation caused by nicotine. It is likely that some amino acids in the transmembrane segments of the β subunits are also important in determining the response of the nAChR to nicotine. Figure 1 shows that there are four amino acid differences between the $\beta 2$ and $\beta 4$ subunits in the M1–M3 region. These nonidentical amino acids in the α subunits and β subunits were the targets for mutagenesis to determine the exact residues that are important in modulating the responses of these nAChRs to nicotine.

Change of $\alpha 4$ Amino Acids to Those Found in $\alpha 3$. Fig. 2 shows that for $\alpha 4\beta 2$ nAChRs nicotine exhibited 100% efficacy and high potency, whereas on $\alpha 3\beta 2$ nAChRs, nicotine exhibited 64% efficacy and lower potency.

When the $\alpha 4$ M1 amino acid 226 was changed from cysteine to phenylalanine, as in $\alpha 3$, the efficacy of nicotine was unchanged (Fig. 3). However, the potency of agonists decreased to more closely resemble the $\alpha 3\beta 2$ nAChR. In the

$\alpha 4$ (C226F) $\beta 2$ nAChR, the EC_{50} values for ACh and nicotine increased 6.9- and 2.5-fold, respectively (Table 2). Thus, this $\alpha 4$ M1 mutation changed potency, not efficacy, and conferred some resemblance to $\alpha 3\beta 2$ nAChRs.

When the $\alpha 4$ M2 leucine 258 was changed to valine, as in $\alpha 3$, the efficacy of nicotine was reduced to 51% (Fig. 3). When the $\alpha 4$ M2 isoleucine 262 was changed to threonine, as in $\alpha 3$, the efficacy of nicotine was reduced to 23%. Thus, both $\alpha 4$ M2 mutations changed efficacy and conferred some resemblance to $\alpha 3\beta 2$ nAChRs.

Changes of $\alpha 3$ Subunit Amino Acids to Those of $\alpha 4$. Changing $\alpha 3$ M2 amino acid 258 from valine to leucine, as in $\alpha 4$, converted nicotine from a partial agonist to a full agonist (Fig. 4; Table 2). Thus, this was the reciprocal effect of the $\alpha 4$ (L258V) mutation that converted nicotine into a partial agonist.

Changing $\alpha 3$ M2 amino acid 262 from threonine to isoleucine, as in $\alpha 4$, did not increase the efficacy of nicotine. Thus, the $\alpha 3$ (T262I) mutation was not reciprocal to the loss in efficacy of the $\alpha 4$ (I262T) mutation.

Voltage Dependence of Nicotine Efficacy. Because mutations in the M2 channel-lining region governed the efficacy of nicotine, the most likely mechanism to explain partial efficacy would be transient occlusion of open channels by nicotine. If this were the mechanism, efficacy should depend on the voltage across the membrane, with more negative holding potentials expected to drive the positively charged nicotine more strongly toward its binding site in the channel, resulting in lower efficacy. The efficacy of nicotine on $\alpha 4\beta 2$ nAChRs was found, as expected, not to depend on the membrane potential (Fig. 5A). For $\alpha 3\beta 2$ nAChRs, the efficacy of nicotine was strongly voltage-dependent (Fig. 5B). Mutating $\alpha 3$ M2 valine 258 to leucine eliminated both the partial efficacy of nicotine and its voltage dependence (Fig. 5B). Conversely, mutating $\alpha 4$ M2 leucine 258 to valine conferred nicotine partial efficacy that was voltage-dependent (Fig. 5A). The fact that the partial efficacy was voltage-dependent ties together two important points regarding mechanism. First, this confirmed that M2 amino acid 258 within the channel was the site responsible for the partial efficacy. For nicotine to act at this site, the drug must move through a portion of the field to reach its blocking site. M2 amino acid 258 and the adjacent amino acids are hydrophobic and may interact with hydrophobic parts of nicotine to slow or stop its passage through the channel. Second, movement through the electric field gave the partial efficacy voltage-sensitivity, because the movement of the charged molecule of nicotine was

TABLE 2
Summary of pharmacological properties of wild-type and mutant nAChRs

AChR		ACh Response			Nicotine Response			Efficacy
α Subunit	β Subunit	Oocytes	EC_{50}	Hill Coefficient	Oocytes	EC_{50}	Hill Coefficient	
			μM			μM		
$\alpha 4$	$\beta 2$	6	3.3 ± 0.6	0.7 ± 0.1	4	3.9 ± 2.6	0.9 ± 0.3	100 ± 15
$\alpha 4$ (C226F), M1	$\beta 2$	6	23 ± 3	0.7 ± 0.1	5	10 ± 1.6	0.9 ± 0.1	100 ± 12
$\alpha 4$ (L258V), M2	$\beta 2$	5	2.1 ± 0.4	0.7 ± 0.1	3	2.8 ± 1.2	0.8 ± 0.2	51 ± 5
$\alpha 4$ (I262T), M2	$\beta 2$	3	3.5 ± 0.9	0.8 ± 0.1	2	0.30 ± 0.1	0.6 ± 0.1	23 ± 1.9
$\alpha 3$	$\beta 2$	2	130 ± 30	1.0 ± 0.2	6	18 ± 5.6	1.1 ± 0.1	64 ± 5.6
$\alpha 3$	$\beta 2$ (I224T, S226L), M1	4	410 ± 120	1.2 ± 0.3	3	190 ± 23	2.0 ± 0.4	65 ± 3.6
$\alpha 3$	$\beta 2$ (V254F), M2	3	54 ± 5	1.1 ± 0.1	3	61 ± 20	0.8 ± 0.2	82 ± 7.7
$\alpha 3$ (V258L), M2	$\beta 2$	3	83 ± 7	1.1 ± 0.1	3	80 ± 4.8	1.0 ± 0.1	100 ± 2.0
$\alpha 3$ (T262I), M2	$\beta 2$	3	48 ± 7	1.0 ± 0.1	4	8.2 ± 1.7	1.0 ± 0.2	55 ± 3.1
$\alpha 3$	$\beta 4$	3	310 ± 40	2.1 ± 0.7	4	330 ± 24	1.5 ± 0.1	76 ± 2.1

altered by the electric field. Even more compelling in support of the mechanism was the fact that the voltage sensitivity of the partial efficacy for both wild-type $\alpha\beta 2$ and $\alpha 4(\text{L258V})\beta 2$

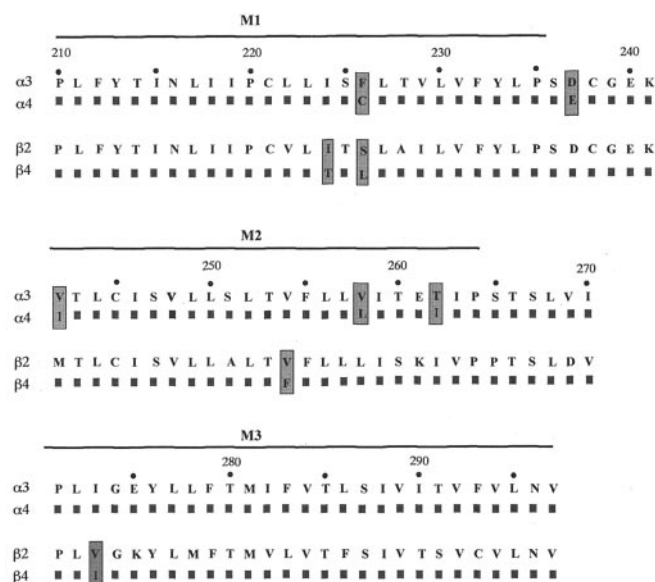


Fig. 1. Comparison of the sequences of human nAChR $\alpha 3$, $\alpha 4$, $\beta 2$, and $\beta 4$ subunits in the first three transmembrane segments from residues 210 to 297 (using the numbering of the α subunits). The full sequences of $\alpha 3$ and $\beta 2$ are shown, and only those amino acids that differ in $\alpha 4$ and $\beta 4$ are shown. In this region, the $\alpha 3$ subunit differs from the $\alpha 4$ subunit by five amino acids, and the $\beta 2$ subunit differs from the $\beta 4$ subunit by four amino acids. A notation is sometimes used in which the amino acid at the N-terminal end nearest the cytoplasmic surface is referred to as the 1' position. In this notation, amino acid 258 is the 17' amino acid, near the extracellular end of the channel.

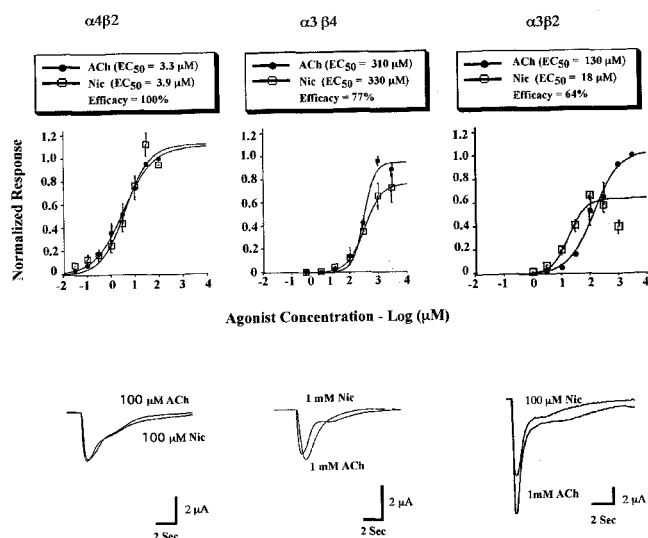


Fig. 2. Comparison of the efficacy and potency of ACh and nicotine on wild-type $\alpha 3\beta 2$, $\alpha 3\beta 4$, and $\alpha 4\beta 2$ nAChRs. Typical responses to the application of ACh and nicotine are shown at concentrations that produce the maximum response, as shown in the concentration-response curve for that nAChR. In the concentration-response curves, the responses to ACh were normalized to the maximum response that was defined as 1. Nicotine responses were also normalized using the maximum response to ACh. This concentration did not cause desensitization when a 3- to 5-min wash was performed between applications. Each point on the dose-response curve represents the normalized mean value from two to six oocytes (see Table 2). Holding potentials were set at -50 mV. Efficacy was defined as the maximum normalized response of nicotine compared with that of ACh. These parameters also apply in Figs. 3, 4, and 6.

was very similar, as evidenced by the slope of the relationship between the efficacy of nicotine and the holding potential for each nAChR. This supported the idea that for these nAChRs, the mechanism was the same, the location of the site for block was the same, and it was determined by amino acid residue 258.

Changing $\beta 2$ Amino Acids to Those Found in $\beta 4$. Nicotine exhibits better efficacy (77%) on $\alpha 3\beta 4$ nAChRs than on $\alpha 3\beta 2$ nAChRs (64%; Fig. 2). Therefore, the efficacy of nicotine seems to depend on both α and β subunits. To investigate the contribution of the β subunit, a double mutation was produced in the $\beta 2$ M1 region that converted isoleucine 224 to threonine and serine 226 to leucine, as in $\beta 4$. The efficacy of nicotine on the $\alpha 3\beta 2(\text{I224T}, \text{S226L})$ nAChR re-

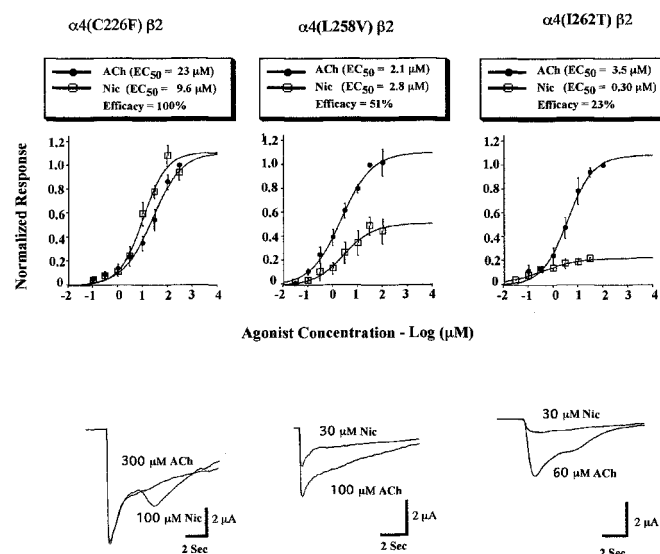


Fig. 3. Comparison of the efficacy and potency of ACh and nicotine on nAChRs that contained mutant $\alpha 4$ subunits coexpressed with wild-type $\beta 2$.

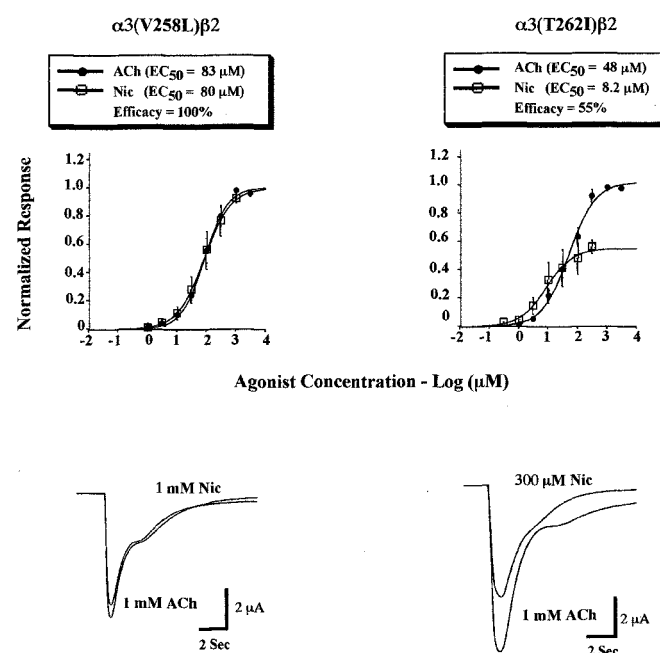


Fig. 4. Comparison of the efficacy and potency of ACh and nicotine on nAChRs that contained mutant $\alpha 3$ subunits coexpressed with $\beta 2$.

mained similar to the wild-type $\alpha 3\beta 2$ nAChR (Fig. 6). However, the potencies of nicotine and ACh decreased 10.6- and 3.1-fold, respectively, to resemble the potencies of these agonists on the $\alpha 3\beta 4$ nAChR (Table 2). Thus, as was seen in the $\alpha 4$ M1 mutation, the β subunit M1 region seems to be important in determining the potency of agonists.

Another mutation was produced in the $\beta 2$ subunit in the M2 region at valine 254, which was converted to phenylalanine, as in $\beta 4$. This mutation increased the efficacy of nicotine to approximately 83%, which is similar to the value obtained for the $\alpha 3\beta 4$ nAChR (77%). Thus, in both α and β subunits, unique amino acids in M1 influence the potency of agonists and unique amino acids in M2 influence the efficacy of nicotine (Table 2).

Differences in Mechanisms of Partial Efficacy of Nicotine and Cytisine. Cytisine characteristically has low efficacy on $\beta 2$ -containing nAChRs compared with $\beta 4$ -contain-

ing nAChRs (Papke and Heinemann, 1993). This low efficacy is thought to reflect intrinsic partial efficacy resulting from the efficiency with which the binding of cytisine triggers channel opening (Figl et al., 1992; Papke and Heinemann, 1993), by contrast with the channel-blocking activity of nicotine. Cytisine was a 25% partial agonist on the $\alpha 3\beta 2$ nAChR and a 60% partial agonist on the $\alpha 3\beta 4$ nAChR. The $\beta 2$ M1 mutant, $\alpha 3\beta 2$ (I224T, S226L) nAChR, exhibited only 2% efficacy for cytisine (Fig. 7). The $\beta 2$ M2 mutant, $\alpha 3\beta 2$ (V254F) nAChR, reduced cytisine efficacy to 11% (Fig. 7). Concentrations of cytisine were chosen such that they produced the maximum response for the nAChR subtype being tested. Thus, the effects of mutations on the efficacy of cytisine do not mirror the pattern seen with nicotine, which is consistent with the mechanism of partial efficacy differing between these two agonists.

Discussion

Our results indicate that the partial efficacy of nicotine on $\alpha 3\beta 2$ nAChRs results from channel block by nicotine at a binding site strongly influenced by M2 amino acid 258, which largely accounts for nicotine channel block of $\alpha 3\beta 2$ but not $\alpha 4\beta 2$ nAChRs. The M2 amino acid 262 of the $\alpha 3$ and $\alpha 4$ subunits with the M2 amino acid 254 of $\beta 2$ and $\beta 4$ subunits also influences this site. The concentrations of nicotine required to produce channel block are larger than those experienced by tobacco users. The greater potencies of nicotine and ACh on $\alpha 4\beta 2$ versus $\alpha 3\beta 2$ and $\alpha 3\beta 2$ versus $\alpha 3\beta 4$ nAChRs were found to result in part from M1 amino acid differences at position 226 of $\alpha 3$ and $\alpha 4$ and positions 224 and 226 of the $\beta 2$ and $\beta 4$ subunits. These M1 amino acids may influence the ease of the conformational change between the resting and open state of the nAChR.

A major component of the ACh binding site is formed by amino acids just N-terminal of M1 in the α and β subunits. This region determines ligand binding affinity and intrinsic efficacy, and it influences potency. Any conformational change triggered by agonist binding must be propagated

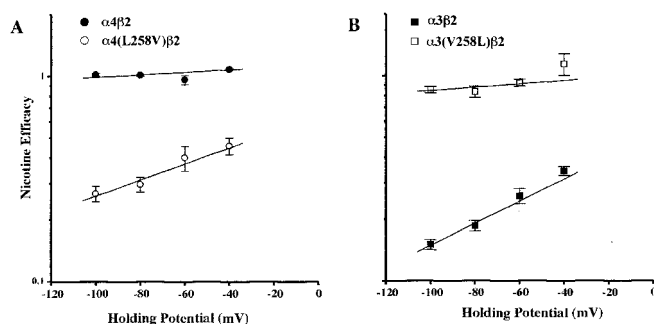


Fig. 5. The efficacy of nicotine is voltage-sensitive on $\alpha 3\beta 2$ and $\alpha 4$ (L258V) $\beta 2$ nAChRs but not on $\alpha 4\beta 2$ or $\alpha 3$ (V258L) $\beta 2$ nAChRs. A, the efficacy of nicotine on wild-type $\alpha 4\beta 2$ nAChRs was voltage-insensitive, whereas the efficacy of nicotine on $\alpha 4$ (L258V) $\beta 2$ nAChRs was voltage-sensitive. B, the efficacy of nicotine on wild-type $\alpha 3\beta 2$ nAChRs was voltage-insensitive, whereas the efficacy of nicotine on $\alpha 3$ (V258L) $\beta 2$ nAChRs was voltage-sensitive. Note the similar voltage sensitivities for the wild-type $\alpha 3\beta 2$ nAChRs and $\alpha 4$ (L258V) $\beta 2$ nAChRs.

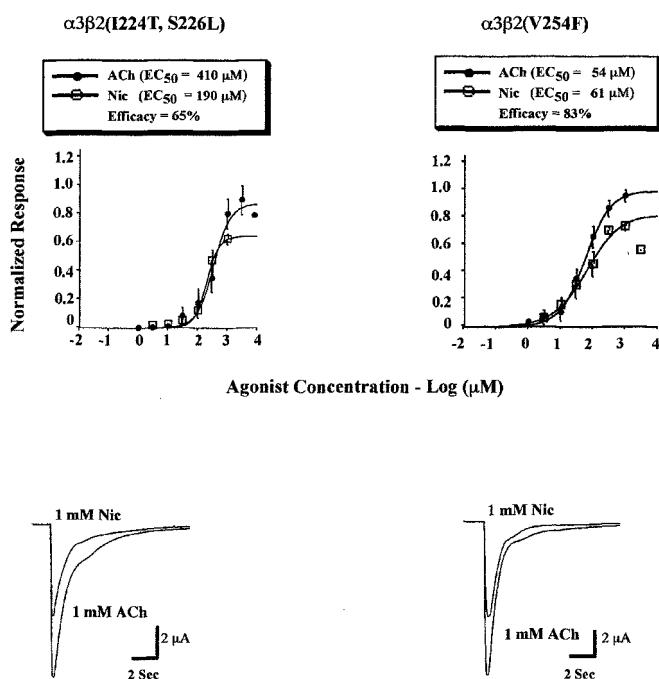


Fig. 6. Comparison of the efficacy and potency of ACh and nicotine on nAChRs that contained mutant $\beta 2$ subunits coexpressed with $\alpha 3$.

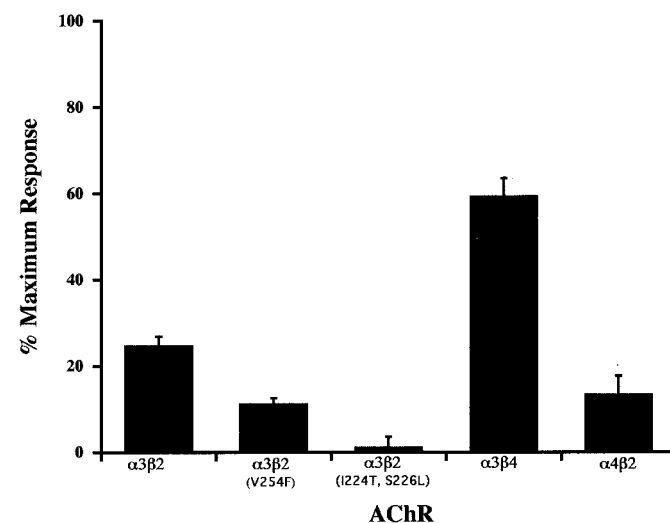


Fig. 7. The efficacy of cytisine relative to ACh was determined for various wild-type and mutant nAChRs. The maximum response to cytisine was normalized to the maximum response to ACh for $\alpha 3\beta 2$ (600 μ M) ($n = 3$), $\alpha 3\beta 4$ (600 μ M) ($n = 3$), $\alpha 3\beta 2$ (I224T, S226L) (1 mM) ($n = 5$), and $\alpha 3\beta 2$ (V254F) (1 mM) ($n = 5$).

from the binding site in the extracellular domain to the channel gate near the cytoplasmic end of the channel in the nAChR. The M1 segments of the subunits should play a major role in propagating this conformational change. The M2 channel-lining domain can also influence potency and efficacy. Some M2 mutations in muscle-type nAChRs make the transition from resting to open state so unstable that it can occur spontaneously or be caused by normally poor agonists (e.g., choline). These experimental and disease-associated mutations may ultimately lead to pathological hyperfunction (e.g., Zhou et al., 1999; De Fusco et al., 2000; Eaton et al., 2000; Orr-Urtreger et al., 2000; Phillips et al., 2001). Although the amino acid exchange experiments reported here are within the confines of normal nAChR subtype sequences, the increased agonist potencies might reflect a similar, but smaller, destabilization of the nAChRs.

Our experiments support the hypothesis that the reduced efficacy of nicotine is due to a low-affinity binding site within the channel of the $\alpha 3\beta 2$ nAChR. Binding to this low-affinity site results in transient occlusion of the channel at high nicotine concentrations, which limits the ability of the channel to conduct cations when activated. Of course, occlusion of nAChR channels by agonists has been well documented (e.g., Sine and Steinbach, 1984; Ogden and Colquhoun, 1985; Macconochie and Knight, 1992). Nicotine has been reported to exhibit partial efficacy on $\alpha 3\beta 4$ nAChRs as a result of channel block (Webster et al., 1999). The positive charge and hydrophobic nature of the agonist itself largely determine the degree of the interaction with agonist within the channel. Nicotine has a polar amine region that allows it to enter the cation channel and a small hydrophobic ring structure that could associate with some of the hydrophobic amino acids within the channel lining. This hydrophobic section could also provide the steric bulk that allows nicotine to obstruct the channel as it stops or moves slowly through the channel. Because nicotine moves through a portion of the membrane electric field to reach its binding site, the affinity of the channel for nicotine is influenced by the membrane holding potential (Woodhull, 1973) and results in the voltage sensitivity of the partial efficacy of nicotine. The significance of the channel block by agonist in the concentration-response relationship is determined by the difference between the concentration needed to activate the channel relative to the concentration of agonist that is needed to occupy the channel binding site to a significant degree. For $\alpha 3\beta 2$ nAChRs, this difference is not great, and nicotine is a partial agonist. But for $\alpha 4\beta 2$ nAChRs, the difference is significant, and the concentrations of nicotine that maximally activate the nAChR do not significantly block the channel. However, when leucine 258 in the $\alpha 4$ subunit is mutated to the $\alpha 3$ -like valine, the affinity of the channel for nicotine increases to a level at which the degree of channel block that occurs at the nicotine concentrations necessary to maximally activate the nAChR becomes significant. Nicotine is a partial agonist on this mutant form of the nAChR.

Reciprocal mutation between $\alpha 4$ and $\alpha 3$ at position 258 produced reciprocal effects in response to nicotine when co-expressed with $\beta 2$. When amino acid 258 was converted from $\alpha 3$ - to $\alpha 4$ -like at this position, nicotine became a full agonist on the $\alpha 3(V258L)\beta 2$ nAChR and was a partial agonist on the $\alpha 4(L258V)\beta 2$ nAChR. In the muscle-type nAChR, the channel-lining regions of M1 and M2 in the $\alpha 1$ subunit have been

determined using the substituted-cysteine accessibility method, SCAM (Akabas et al., 1994; Akabas and Karlin, 1995). The equivalent valine in the $\alpha 1$ subunit of the muscle nAChR is not accessible to the SCAM labeling reagents. Therefore, it is likely (but not certain) that this valine is also inaccessible to the aqueous lumen of the channel in $\alpha 3$ and $\alpha 4$ subunits. To fully understand the contributions of relevant $\alpha 3$ and $\beta 2$ amino acids in forming a low-affinity binding site for nicotine in the channel would require structural determinations of the channel. The SCAM technique has been used to model the channel blocking site for the lidocaine derivative QX-222 in the muscle nAChR (Pascual and Karlin, 1998), and it was found to lodge deep in the channel, well beyond the equivalent of V258. Thus, V258 of $\alpha 3$ and $\alpha 4$, which greatly influences channel blockage by nicotine, is surprisingly close to the extracellular lumen of the channel. There are two equivalent α/β interfaces in the lumen of an $(\alpha)_2(\beta)_3$ nAChR channel, and binding of two nicotine molecules rather than just one might be associated with channel block in a wider part of the channel. Nicotine exhibits 100% efficacy on $\alpha 6\alpha 3\beta 2$ nAChRs (Kuryatov et al., 2000b). Thus, the presence of only one $\alpha 3\beta 2$ interface may be insufficient to permit channel block by nicotine. Note that the M2 sequences of $\alpha 6$ and $\alpha 3$ subunits are identical, but that they differ in two M1 amino acids and three M3 amino acids. $\alpha 6$ has a methionine instead of $\alpha 3$ leucine 211 near the extracellular end of the channel, which might disrupt nicotine binding to M2 at this end of the channel.

Both the α and β subunits modulate the effects of nAChR agonists and antagonists. The M2 254 mutation that converted the $\beta 2$ valine residue to a $\beta 4$ -like phenylalanine slightly increased the efficacy of nicotine. In the muscle-type nAChR, the M1 and M2 channel-lining regions of the $\beta 1$ subunit have been determined using the SCAM assay (Zhang and Karlin, 1997, 1998). The equivalent valine in the $\beta 1$ subunit is accessible to the labeling reagents from the channel lumen. Therefore, this residue could be similarly accessible in the $\beta 2$ subunits. The position of residue numbered 254 in the $\beta 2$ subunit would be approximately one α helical turn below the efficacy determining position 258 in the $\alpha 3$ or $\alpha 4$ subunit, if these were both aligned helices (which may not be the case).

The potency of ACh and nicotine on nAChRs that contained M1 mutations changed compared with the potency of these agonists on the wild-type nAChRs. However, the efficacy of nicotine did not change. These positions include residue 226 in the $\alpha 3$ or $\alpha 4$ subunit and residues 224 and 226 in the $\beta 2$ or $\beta 4$ subunit. These residues may alter the relative stabilities of the resting and activated conformations of the nAChRs by indirectly altering the way agonist binding favors the transition to the active conformation. The equivalent positions in the $\alpha 1$ subunit and the $\beta 1$ subunit of the muscle nAChR were not channel-lining residues in the SCAM assay (Zhang and Karlin, 1997).

The M1 mutations cause an approximate shift in EC_{50} value from one wild-type nAChR to the wild type of nAChR that is contributing the mutant amino acid. For example, the $\alpha 4(C226F)\beta 2$ nAChR has an EC_{50} value for ACh similar to wild-type $\alpha 3\beta 2$ nAChR. It seems unlikely that mutations in M1 would alter an agonist-binding site in the extracellular domain. It seems more likely that mutations in M1 would alter the ease of the transition from the resting to activated

conformation, a change that is likely to involve many parts of the protein. In a model of channel closure that involves a change in conformation of five bent M2 α -helical segments (Unwin, 2000), local interactions among the side chains of M1 and M2 could be important in determining the stability of different states of the nAChR. It has also been shown that some M2 amino acids influence potency (Eaton et al., 2000; Orr-Urtreger et al., 2000; Phillips et al., 2001).

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