Regions of β2 and β4 Responsible for Differences between the Steady State Dose-Response Relationships of the α3β2 and α3β4 Neuronal Nicotinic Receptors

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ABSTRACT We constructed chimeras of the rat β2 and β4 neuronal nicotinic subunits to locate the regions that contribute to differences between the acetylcholine (ACh) dose-response relationships of the α3β2 and α3β4 receptors. Expressed in Xenopus oocytes, the α3β2 receptor displays an EC50 for ACh ~20-fold less than the EC50 of the α3β4 receptor. The apparent Hill slope (n_app) of α3β2 is near one whereas the α3β4 receptor displays an n_app near two. Substitutions within the first 120 residues convert the EC50 for ACh from one wild-type value to the other. Exchanging just β2:104–120 for the corresponding region of β4 shifts the EC50 of ACh dose-response relationship in the expected direction but does not completely convert the EC50 of the dose-response relationship from one wild-type value to the other. However, substitutions in the β2:104–120 region do account for the relative sensitivity of the α3β2 receptor to cytisine, tetramethylammonium, and ACh. The expression of β4-like (strong) cooperativity requires an extensive region of β4 (β4:1–301). Relatively short β2 substitutions (β2:104–120) can reduce cooperativity to β2-like values. The results suggest that amino acids within the first 120 residues of β2 and the corresponding region of β4 contribute to an agonist binding site that bridges the α and β subunits in neuronal nicotinic receptors.

INTRODUCTION

Both the α and non-α subunits of the nicotinic acetylcholine receptor (nAChR) play a role in the activation of the receptor by agonist (reviewed in Deneris, Connolly, Rogers, and Duvoisin, 1991; Karlin, 1993; Papke, 1993). Although many studies show that agonist binds to the α subunit (reviewed in Karlin, 1993), present opinion is divided about the possibility of an agonist binding site at the α/non-α subunit interface (Cockcroft, Osguthorpe, Barnard, and Lunt, 1990; Karlin, 1993; Unwin, 1993). The evidence for an α-only binding site is that 9-Å images of Torpedo nAChRs...
reveal a unique cleft within the α subunit that could act as an agonist binding pocket (Unwin, 1993). However, there is no evidence that agonists actually bind within this cleft and contact only α-subunit residues. On the other hand, previous experiments show that (a) the muscle-type α subunit (α1) binds nicotinic agonists with appreciable affinities only if it is complexed with a non-α subunit (Blount and Merlie, 1988) and (b) the competitive antagonist d-tubocurarine (dTC) photolabels residues on the γ and δ subunits of Torpedo nAChRs (Pedersen and Cohen, 1990; Chiara and Cohen, 1992), which is strong evidence for a direct participation of the non-α subunits in an agonist binding site. However, dTC is a larger molecule than ACh and may not contact exactly the same residues that ACh does. Large quantities of purified neuronal nAChRs are not available for direct biochemical and structural analyses of potential agonist binding sites. We have exploited the fact that α3β2 and α3β4 neuronal nAChRs expressed in Xenopus oocytes display different relative sensitivities to the ganglionic agonists, cytisine (CYT), tetramethylammonium (TMA), acetylcholine (ACh) and nicotine (Leutje and Patrick, 1991; Figl, Cohen, Quick, Davidson, and Lester, 1992), and different dose-response relationships for ACh (Couturier, Erkman, Valera, Rungger, Bertrand, Boulter, Ballivet, and Bertrand, 1990; Cachelin and Jaggi, 1991; Leutje, Piattoni, and Patrick, 1993; Wong, Mennerick, Clifford, Zorumski, and Isenberg, 1993), to locate the residues of β2 and β4 involved in agonist activation of the receptor.

In an earlier study on coexpressed α3 and β subunits, we used β4-β2 chimeras. Extending the β4 NH2-terminal region of a chimera from residues β4:1-105 to β4:1-122 (Fig. 1) changed the CYT/ACh and TMA/ACh response ratios from α3β2-like (β2-like) to α3β4-like (β4-like) levels (Figl et al., 1992). These data suggested that β4:106-122, and the corresponding region of β2 (β2:104–120), contributes to an agonist binding site that bridges the α and non-α subunits. To test this hypothesis, we have extended the earlier studies with (a) more chimeric and mutated β subunits and (b) electrophysiological measurements of the agonist dose-response relationship, which we summarize by the half-maximal agonist concentration (EC50) and Hill coefficient (napp). The results show that the region of β4 that converts the EC50 for ACh from a β2-like to a β4-like value overlaps the previously localized region that converts the CYT/ACh response ratios of β4 NH2-terminal chimeras from β2-like to β4-like values (Figl et al., 1992) and suggest that an important component of the agonist binding site lies within the first 120 residues of β2 and the corresponding region of β4. Some of our results have been reported previously in abstract form (Figl, Cohen, Gollub, Davidson, and Lester, 1993).

METHODS
Construction of the β Chimeras and Mutants

We created chimeras between the β2 and β4 subunits by taking advantage of shared restriction sites in the two subunits and using the polymerase chain reaction (PCR) to generate overlapping cDNA fragments (Higuchi, 1990) from the two subunits. Fig. 1 shows an alignment of β2 and β4 amino-acid sequences. Most of the chimeras contained the NH2-terminal region from one β subtype joined to the succeeding COOH-terminal residues from the other β subtype (NH2-terminal chimeras). We refer to the NH2-terminal chimeras in the text by
FIGURE 1. The aligned amino acid sequences of the rat β2 and β4 neuronal nAChR subunits. The boxes outline identical residues and the shading denotes homologous residues. Residue 1 begins after the termination of the leader sequences (not shown). The arrows above the β4 sequence point to the transitions in the β4 NH₂-terminal chimeras between the β4 and β2 sequence. The arrows beneath the β2 sequence point to the transitions in the β2 NH₂-terminal chimeras between the β2 and β4 sequence. The numbers above (below) the arrows are the last residues in the β4 (β2) NH₂-terminal regions of the chimeras. The lines beneath the β2 sequence denote the transmembrane regions M1–M4.
transition points between the two types of β subunits. For example, β2(229)β4 is a chimera containing the first 229 residues of β2 joined to residues 232–475 of β4 (see Fig. 1). Chimeras containing internal substitutions are labeled by the end points of the substitution. For example, β4 β2(104–120)β4 is a chimera in which we substituted β2:104–120 for residues 106–122 of the β4 subunit (these labels have been shortened to β2(104–120) and β4(106–122) in Figs. 7 and 8).

To create the β2(229)β4 and β4(231)β2 chimeras, we excised the cDNA fragments for residues β2:1–229 and β4:1–231 from the wild-type subunits with EcoRI and BbsI, subcloned the excised fragments into the complementary restricted plasmid from the opposite β subunit, and sequenced the region around the β4/β2 transition to check the final product. The remaining chimeras were constructed in three steps: (a) we synthesized two or more separate PCR fragments; (b) we annealed and amplified the PCR fragments; and (c) we inserted the annealed fragment into the appropriate restriction sites in the wild-type plasmid. We normally introduced a silent mutation into the chimera during the initial PCR reaction to allow us to check the final product by restriction analysis. In addition, all the chimeras were verified by DNA sequencing. We made point mutations using the Altered Sites Kit (Promega Corp., Madison, WI) and verified the mutations by restriction analysis and DNA sequencing. Point mutations are denoted in the text by subscripts that contain the single letter code for the original residue, followed by its position in the sequence, and the residue to which it was mutated. We synthesized mRNA in vitro using the MEGAscript Kit (Ambion, Austin, TX) or a previously published method (Guastella, Nelson, Nelson, Czyzyk, Keynan, Midel, Davidson, Lester, and Kanner, 1990) and checked the size of the synthesized mRNA by agarose gel electrophoresis.

Oocyte Expression

Stage V–VI Xenopus oocytes were isolated as described previously (Quick and Lester, 1994) and injected with mRNA in a stoichiometric ratio for α:β of 2:3. The amount of mRNA injected (< 25 ng/subunit) was adjusted to maintain a maximum response (I_{max}) of ≤ 10 μA at −50 mV. Before recording, the oocytes were incubated for 2–7 d at 18°C in a modified Barth’s solution supplemented with 50 μg/ml gentamicin, 2.5 mM pyruvate, and 5% horse serum (Quick and Lester, 1994).

ElectrophysiologicalRecordings

The oocytes were voltage clamped at −50 mV with two, 3 M KCl-filled microelectrodes made from thick-walled borosilicate glass (model BF150-86-10, Sutter Instrument Co., Novato, CA) and an AxoClamp 2A voltage clamp (Axon Instruments, Foster City, CA). During the experiments, we perfused the recording chamber (~1.5 ml vol) with 98 mM NaCl, 1 mM MgCl₂, and 5 mM HEPES (pH 7.4) at room temperature (20–24°C) at 10–20 ml/min. We omitted Ca²⁺ from the recording solution because the entry of Ca²⁺ through neuronal nAChRs can activate the endogenous Ca²⁺-activated Cl⁻ current of the oocyte (Vernino, Amador, Leutje, Patrick, and Dani, 1992).

We applied agonists to the oocytes by pinching-off the outflow of a U tube (Bormann, 1992) with a solenoid-activated pinch valve (Cole-Palmer, Niles, IL) under computer control. The time constant of the approach to a steady state response (τ_{ss}) was 0.4–0.5 s at slowly desensitizing agonist doses. To check the effective [agonist] reaching the oocyte from the U tube, we applied a slowly desensitizing dose of ACh (1 μM) to an oocyte expressing α3β2 using the bath and U tube perfusion systems. Bath and U tube application of 1 μM ACh produced the same steady state response. Endogenous muscarinic responses were rare and easily identified by their oscillatory nature and delayed onset after agonist application from the U tube. We did not use oocytes exhibiting muscarinic responses.
Collection and Analysis of the Dose-Response Data

We recorded the responses to 6–11 different agonist doses to measure the dose-response relationships for individual oocytes. The agonist was applied in 6-s pulses separated by a 2–3-min rinse with control saline. The responses were filtered at 20 Hz and digitized at a sampling frequency of 50 Hz using an MS-DOS computer equipped with pCLAMP V5.5 software (Axon Instruments).

We fit the dose-response data for individual oocytes to the Hill equation,

\[
I_{\text{peak}} = \frac{I_{\text{max}}}{1 + \left(\frac{EC_{50}}{[A]}\right)^{n_{\text{app}}}},
\]

where \(I_{\text{peak}}\) was the peak response at agonist concentration \([A]\), using weighted nonlinear least-squares regression. To reduce the interdependence of the parameters \(I_{\text{max}}, EC_{50},\) and \(n_{\text{app}},\) we allowed only the \(I_{\text{max}}\) and the \(EC_{50}\) to vary when fitting to the Hill equation. The \(n_{\text{app}}\) was fixed at the mean slope of the Hill plots for the receptor subtype being fit. The \(n_{\text{app}}\) was determined (a) from the Hill plot by linear regression of \(\log(I_{\text{peak}}/(I_{\text{max}} - I_{\text{peak}}))\) against \(\log([\text{agonist}])\) where \(I_{\text{max}}\) was the maximum observed response and (b) from the log–log plot by regression at the foot of the dose-response relationship. The correlation between these two measurements of the \(n_{\text{app}}\) was 0.89 for the 26 receptor types discussed in the text. Regression of the log–log slope against the corresponding Hill slope predicted a log–log slope of 1.1 for a Hill slope of 1.0 and a log–log slope of 1.9 for a Hill slope of 2.0. We preferred the estimates of the \(n_{\text{app}}\) from the Hill plot because (a) the slope of the Hill plot was based on more points per oocyte than the initial log–log slope measurements, (b) log–log slope measurements tend to underestimate the true \(n_{\text{app}},\) (c) the Hill plot included more data points with a higher signal/noise ratio than the initial log–log plot, and (d) the \(n_{\text{app}}\)'s from the Hill plot generally fit the dose-response relationship better than the \(n_{\text{app}}\)'s from the log–log plots. The \(n_{\text{app}}\)'s reported in the text are from Hill slopes rather than from log–log slopes unless stated otherwise. We weighted the residuals by \(1/\sqrt{I_{\text{peak}}}\) for the fit to the Hill equation because the variance of \(I_{\text{peak}}\) at the high end of the dose-response relationship was larger than the variance of \(I_{\text{peak}}\) at the low end. Repeated measurements on a single oocyte showed that long-term desensitization reduced the \(I_{\text{max}}\) but did not affect the \(EC_{50}\) or \(n_{\text{app}}\).

To compare measurements of the \(EC_{50}\) with widely different variances, we transformed the \(EC_{50}\)'s to log values. Unless otherwise stated, the measurement errors reported in the text are standard deviations (SDs) and the SDs for the \(EC_{50}\)'s given in the text are for untransformed data. To construct normalized dose-response relationships for the receptors, we normalized the individual dose-response relationships to the fitted \(I_{\text{max}}\)'s and pooled the resulting data. We then refit the Hill equation allowing only the \(EC_{50}\) to vary. The error reported for the normalized \(EC_{50}\) measurements is the standard error of estimate (SEE).

Correction of the Peak Response for Desensitization

Rapid desensitization reduces the peak response because some receptors desensitize while the [agonist] is still rising around the oocyte. We used the following three-state scheme (Scheme 1) to calculate depression of the peak response by rapid desensitization,

\[
C \xrightarrow{k_1} O \xrightarrow{k_2} D,
\]

SCHEME 1
where C, O, and D are the closed, open, and desensitized states of the receptor, \( k_1 \) is the rate constant for the \([ACh]\) to reach a steady state around the oocyte, and \( k_2 \) represents the rate constant for desensitization. We obtained \( k_1 \) directly from the rate constant of the rising phase of the response with 1 μM ACh and \( k_2 \) directly from the rate constant of the decay of the response within the agonist pulse (in the Results section, \( 1/k_1 \) and \( 1/k_2 \) are termed \( \tau_{on} \) and \( \tau_{down} \)). The relationship between the peak amplitude of the observed response (\( I_{\text{peak}} \)) and the peak amplitude of the calculated response (\( I_{\text{calc}} \)) is,

\[
I_{\text{calc}} = \frac{I_{\text{peak}}}{k_1 - k_1 \left( \frac{k_1}{k_2} \right) \left( \frac{k_1}{k_2} \right)}
\]

At high \([ACh]\)'s, the decay of the response consisted of two exponential components. These components were corrected separately using Eq. 1 and then summed to obtain the final calculated peak response.

RESULTS

The \( \beta \) Subunit Affects the Agonist Dose-Response Relationship

Consistent with previous data (Couturier et al., 1990; Leutje et al., 1993; Wong et al., 1993), we found that the \( \alpha_3 \) subunit profoundly affects the sensitivity of neuronal nAChRs to ACh. Expressing \( \alpha_3 \) with \( \beta_2 \), instead of with \( \beta_4 \), shifted the ACh dose-response relationship at -50 mV considerably to the left and reduced its slope (Fig. 2). The traces in Fig. 2, A and B, are typical ACh responses of \( \alpha_3 \beta_2 \) (Fig. 2 A) and \( \alpha_3 \beta_4 \) (Fig. 2 B) from oocytes with similar maximum responses. We fit the ACh

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**Figure 2.** (opposite) Expressing \( \alpha_3 \) with \( \beta_2 \), rather than with \( \beta_4 \), reduces the EC\(_{50}\) and apparent Hill coefficient (\( n_{\text{app}} \)) of the ACh dose-response relationship. (A) Responses of \( \alpha_3 \beta_2 \) to 1–300 μM ACh. The numbers are the \([ACh]\) in μM. The square pulse above the traces is the duration of the application of ACh from the U tube. (B) Responses of \( \alpha_3 \beta_4 \) to 20–1,000 μM ACh. (C) Dose-response plots for \( \alpha_3 \beta_2 \) and \( \alpha_3 \beta_4 \). The symbols denote different oocytes expressing \( \alpha_3 \beta_2 \) (filled circles, open circles, filled inverted triangles, open inverted triangles, filled squares) or \( \alpha_3 \beta_4 \) (open squares, filled upright triangles, open upright triangles). The lines are fits to the Hill equation (see Methods). The EC\(_{50}\)’s and the \( I_{\text{max}} \)’s are 17 μM and 1,863 nA (filled circles), 13 μM and 1,757 nA (open circles), 15 μM and 2,056 nA (filled inverted triangles), 13 μM and 1,461 nA (hollow inverted triangles), 13 μM and 1,498 nA (filled squares), 216 μM and 2,944 nA (open squares), 232 μM and 2,061 nA (filled upright triangle), and 209 μM and 2,100 nA (open upright triangle). Voltage = -50 mV. (D) Hill plots for the peak ACh responses (\( I_{\text{peak}} \)) of \( \alpha_3 \beta_2 \) and \( \alpha_3 \beta_4 \). \( I_{\text{max}} \) is the maximum observed \( I_{\text{peak}} \). The symbols denote different oocytes; \( \alpha_3 \beta_2 \) (filled circles, hollow circles), \( \alpha_3 \beta_4 \) (filled inverted triangles, hollow inverted triangles, filled squares). Overlap obscures some of the data. The \( n_{\text{app}} \)’s are 0.9 (filled circles), 1.0 (open circles), 2.2 (filled inverted triangles), 2.3 (inverted triangles), and 2.2 (filled squares). (E) Log-log plots of the foot of the ACh dose-response relationship show that the slope of the \( \alpha_3 \beta_4 \) log-dose-log response relationship is ~twice that of \( \alpha_3 \beta_2 \). The symbols represent data from six oocytes (each oocyte is represented by a different symbol). The lines are regression lines fit to the log-log data. The slopes for \( \alpha_3 \beta_4 \) are 2.0 (filled circles), 2.0 (open circles), and 1.8 (filled inverted triangles); the slopes for \( \alpha_3 \beta_2 \) are 1.1 (hollow inverted triangles), 1.0 (filled squares), and 1.0 (hollow squares).
dose-response relationships for α3β2 and α3β4 to the Hill equation (Fig. 2 C) using the n_{app}’s for α3β2 and α3β4 determined from Hill plots of the ACh responses (Fig. 2 D). Similar to previous results (Couturier et al., 1990; Leutje et al., 1993; Wong et al., 1993), the EC_{50} for ACh was 11 ± 4 μM (mean ± SD, n = 29 oocytes) for the rat α3β2 receptor and 219 ± 12 μM (n = 3) for the rat α3β4 receptor. The n_{app} for α3β2 was 1.1 ± 0.2 (n = 29) and the n_{app} for α3β4 was 2.2 ± 0.1 (n = 3). Fig. 2 D shows Hill plots for the two receptors. Measurements of the n_{app} from the initial slope of a log-log plot of the dose-response relationship (where desensitization and agonist
self-block are minimal) gave similar, but slightly smaller, \( n_{\text{app}} \) s for \( \alpha_3\beta_2 \) (0.9 ± 0.1, \( n = 11 \)) and \( \alpha_3\beta_4 \) (1.9 ± 0.1, \( n = 3 \)). Fig. 2E shows examples of the initial portions of the log(dose)–log(response) relationships for the two wild-type receptors. The agreement of these measurements shows that the difference between the \( n_{\text{app}} \) s of \( \alpha_3\beta_2 \) and \( \alpha_3\beta_4 \) was not due to desensitization or open-channel block by the agonist. Injecting equal amounts of mRNA generally gave much larger maximal ACh responses for \( \alpha_3\beta_4 \) than for \( \alpha_3\beta_2 \) (~10-fold) but we did not study these differences further.

The dose-response relationships of \( \alpha_3\beta_2 \) and \( \alpha_3\beta_4 \) for TMA displayed differences comparable to, but less pronounced than, those for ACh (Fig. 3). Fig. 3A and B, shows sample responses of \( \alpha_3\beta_2 \) (Fig. 3A) and \( \alpha_3\beta_4 \) (Fig. 3B) to TMA. Rapid desensitization is less pronounced for both wild-type receptors with TMA as the agonist. The EC\(_{50}\) of \( \alpha_3\beta_2 \) for TMA (76 ± 11 \( \mu \)M, \( n = 7 \)) was fivefold smaller than that of \( \alpha_3\beta_4 \) (424 ± 94 \( \mu \)M, \( n = 6 \)) and the \( n_{\text{app}} \) of \( \alpha_3\beta_2 \) for TMA (1.4 ± 0.1, \( n = 7 \)) determined from Hill plots of the dose-response data was significantly smaller than the \( n_{\text{app}} \) of \( \alpha_3\beta_4 \) (2.0 ± 0.2, \( n = 6 \)). As with ACh, the \( \beta_2 \) subunit shifts the TMA dose-response considerably to the left (Fig. 3C). Thus, the \( \beta \) subunit affects the activation of neuronal nAChRs by an agonist that contains only the quaternary ammonium portion of the ACh molecule; and the effects of the \( \beta \) subunit on the agonist dose-response relationship persist even with an agonist that desensitizes the receptor more slowly than ACh.

**Rapid Desensitization Fails to Account for Differences in the EC\(_{50}\)'s of \( \alpha_3\beta_2 \) and \( \alpha_3\beta_4 \)**

The response of \( \alpha_3\beta_2 \) to ACh (Fig. 2A) desensitized more quickly than the response of \( \alpha_3\beta_4 \) to ACh (Fig. 2B). The time constant \( \tau_{\text{on}} \) for activation of the \( \alpha_3\beta_2 \) response to 1 \( \mu \)M ACh to reach a steady state was 0.4–0.5 s. The time constant \( \tau_{\text{desen}} \) for the desensitization of the \( \alpha_3\beta_2 \) response decreased from 5–7 s at 3 \( \mu \)M ACh to 0.6–0.8 s at 300 \( \mu \)M ACh. Desensitization of the \( \alpha_3\beta_2 \) response to 300 \( \mu \)M ACh was biphasic with an additional slow time constant of 5–6 s. The \( \alpha_3\beta_4 \) receptor displayed little desensitization during the 6-s agonist pulse. The \( \tau_{\text{desen}} \) for \( \alpha_3\beta_4 \) was 5–6 s at 1–2 mM ACh and was too slow to measure at 20–500 \( \mu \)M ACh.

We corrected the peak ACh response of the wild-type receptors for apparent desensitization (see Methods) and fit the corrected data to the Hill equation to determine how much of the difference in the EC\(_{50}\)'s could be due to a difference in the rate of desensitization. Correcting the peak response of \( \alpha_3\beta_2 \) for apparent desensitization left \( n_{\text{app}} \) unchanged but increased the EC\(_{50}\) of \( \alpha_3\beta_2 \) for ACh twofold. Desensitization of \( \alpha_3\beta_4 \) did not significantly affect the ACh dose-response relationship. Thus, allowing for the potential effects of desensitization, the EC\(_{50}\) of \( \alpha_3\beta_4 \) for ACh was still 10-fold larger than the EC\(_{50}\) of \( \alpha_3\beta_2 \). The differences in desensitization accounted for such a small fraction of the differences between the EC\(_{50}\)'s of the wild-type receptors that we decided not to attempt to correct the amplitudes of the peak responses. We were unable to resolve desensitization occurring on a time scale faster than 1 s (Maconochie and Knight, 1992) using our drug application system (see
Such desensitization would produce an apparent decrease in the EC₅₀ of the dose-response relationship in our experiments.

**NH₂-Terminal Substitutions of 105 or More β₄ Residues Increase the EC₅₀ for ACh**

Substitutions of 94 or fewer β₄ residues had only small effects on the EC₅₀ for ACh (Fig. 4 A); the EC₅₀'s of the α3β₄(16)-β₂, α3β₄(81)-β₂, and α3β₄(94)-β₂ chimeras for ACh (13–18 μM) were within a factor of two of the EC₅₀ of α3β₂ for ACh. However, if the β₄ NH₂-terminal region of the chimeras was extended from 94 to 109 residues, the EC₅₀ for ACh increased dramatically to a β₄-like value. Fig. 4 B presents data for the interesting region between residues β₄:94 and β₄:109 and shows the rightward shift of the normalized ACh dose-response relationships as more β₄ residues are
included. All the β4 NH2-terminal chimeras with 113 or more β4 NH2-terminal residues displayed β4-like EC50's for ACh (> 10-fold larger than that of α3β2). Thus, the β4/β2 residue substitutions necessary for β4-like ACh sensitivity in the β4/β2 chimeras lie in the first 113 residues of β4.

**Figure 4.** (A) Substituting 105 or more β4 NH2-terminal residues for the corresponding region of β2 increases the EC50 for ACh. The horizontal axis is the number of β4 NH2-terminal residues in the chimera: zero for α3β2 and 475 for α3β4. The symbols are the mean values for the two wild types and 14 β4/β2 chimeras (n = 2–29 oocytes). Overlap obscures some of the data. The dashed lines are 1 ± SD. The means and SDs for the EC50's were computed from log transformed data. The vertical line denotes the beginning of M1. (B) The addition of two residue substitutions in the β4:95–109 sequence (see Fig. 1) shifts the ACh dose-response relationship dramatically to the right. The symbols are the mean normalized responses ± SD (see Methods) for α3β4(94)β2, α3β4(105)β2, and α3β4(109)β2. The lines are fits to the Hill equation (see Methods). The EC50's for ACh are 16 ± 1 μM (n = 9), 60 ± 4 μM (n = 4), and 338 ± 23 μM (n = 6), respectively (±SEE). (C) Substituting ≥301 β4 NH2-terminal residues increases the napp for ACh to ≥2.0.

There are only two β4/β2 residue substitutions in β4:95–109 (see Fig. 1). The addition of these two substitutions increased the EC50 of the β4 NH2-terminal chimeras from β2-like to β4-like levels. Therefore, we made the site-directed mutations β2M101T/F106V to change just these residues in the β2 subunit. The EC50 of
the mutated receptor for ACh (19 ± 4 µM, n = 5) was within a factor of two of the value for αβ2 (11 ± 4 µM). Thus, the increase in the EC50 of αβ4(109)-β2 over the EC50 of αβ4(94)-β2 depended on residue substitutions within the first 94 residues of β4 in addition to the two substitutions β2:Met101→β4:Thr103 and β2:Phe106→β4:Val108. Previous experiments (Chiara et al., 1992; O’Leary, Filatov, and White, 1994) also suggest that a tryptophan (γ:Trp55, δ:Trp57) in the first 60 residues of the non-α subunits contributes to the agonist binding sites of the Torpedo nAChR.

We also made the mutation αβ4(61)SZ8N to determine if the loss of a potential glycosylation site at δ:Asn26 was responsible for the slight decrease in the EC50 of αβ4(61)-β2 below that of αβ2. The EC50 (6 ± 3 µM, n = 4) and napp (0.8 ± 0.1, n = 4) of αβ4(61)SZ8N-β2 for ACh were not significantly different from the EC50 (5 ± 1 µM, n = 6) and the napp (1.0 ± 0.1, n = 6) of αβ4(61)-β2. Thus, the removal of a potential glycosylation site at β2:Asn26 was not responsible for the effect of αβ4(61)-β2 on the EC50 for ACh.

*Increasing the napp to β4-like Values Requires an Extensive Region of β4*

The napp of the β4-β2 chimeras showed a more gradual dependence on the β4/β2 transition point than the EC50. The napp’s of these chimeras fell into three categories, β2-like (napp = 1.0–1.2), intermediate (napp = 1.6), and β4-like (napp = 2.0–2.2). The chimeras containing 109 or less β4 NH2-terminal residues exhibited β2-like napp’s; the chimeras containing 109–231 β4 NH2-terminal residues exhibited intermediate napp’s except for αβ4(122)-β2 (napp = 1.2 ± 0.2, n = 2); and the chimeras containing 301 or more β4 NH2-terminal residues exhibited β4-like napp’s. The β2M101T,F106V mutation did not affect the napp for ACh.

We are not surprised by the differences between the regions of β4 that affect the EC50 and those that affect the napp. Areas of contact between the subunits outside the agonist binding site may affect the cooperativity of a multisubunit receptor (Koshland, Nemethy, and Filmer, 1966). Nevertheless, the overlap between the β4 residues that increase the EC50’s of the β4-β2 chimeras and the residues that increase the napp from β2-like to intermediate values suggests that β4:106–122 and β2:104–120 may be areas where the α and β subunits contact each other. The results for the β4 NH2-terminal chimeras also suggest that these subunits contact each other somewhere between the end of the first putative transmembrane domain M1 and the initial portion of the M3–M4 intracellular loop. However, our data do not allow us to exclude the alternative hypothesis that these residue substitutions produce a conformational change in the receptor that affects cooperativity indirectly.

*Substituting β2:1–120 for the Corresponding Region of β4 Reduces the EC50 to a β2-like Value*

We constructed a number of β2-β4 chimeras to determine the region of the β2 subunit required to reduce the EC50 of the chimeras to β2-like values (Fig. 5 A). Similar to results for the β4-β2 chimeras, substituting the first 120 residues of β2 for the corresponding region of β4 reduced the EC50 for ACh to 16 ± 8 µM (n = 3) which resembles the EC50 of αβ2 (11 ± 4 µM). Fig. 5 B shows the effect of this substitution on the ACh dose-response relationship. Substituting the first 92 residues of β2 also reduced the EC50 for ACh (Fig. 5 A). Thus, residue substitutions in the first
92 amino acids of β2 do affect the sensitivity of the receptor to agonist but this effect is masked in the β4 NH2-terminal chimeras.

There is an anomalous member of the β2-β4 series; the EC50 of αβ2(229)β4 for ACh was close to that of αβ4 (Fig. 5 A). This anomalous EC50 does not seem to be due to a disruption of the agonist binding site. Previous data show that this chimera displays a β2-like affinity for the irreversible competitive inhibitor, neuronal bungarotoxin (Papke, Duvoisin, and Heinemann, 1993), and we found that αβ2(229)β4 exhibits a clear β2-like insensitivity to CYT relative to ACh (Fig. 6).

Thus, the results for the β4 and β2 NH2-terminal chimeras show that β2/β4 substitutions in the first 120 residues of β2 and the first 113 residues of β4 are sufficient to convert the EC50 for ACh from one wild-type value to another.

Figure 5. (A) β2 NH2-terminal substitutions have a more complex effect on the EC50 for ACh than β4 NH2-terminal substitutions. The symbols are mean values for the two wild types and five β2 NH2-terminal chimeras (n = 3–29). The dashed lines are ± SD. The vertical line denotes the beginning of M1. (B) Substituting β2:1–120 for the corresponding region of β4 reduces the EC50 to a β2-like value. The symbols are the normalized responses ± SD. The lines are fits to the Hill equation. The EC50’s for αβ2(120)β4 and αβ4 are 15 ± 3 μM (n = 3) and 215 ± 8 μM (n = 3) (± SEE). (C) Substituting 93 or more β2 NH2-terminal residues for the corresponding regions of β4 reduces the napp for ACh to β2-like values.
Short $\beta_2$ NH$_2$-Terminal Substitutions Disrupt the Cooperativity of the Receptor

$\beta_2$ NH$_2$-terminal sequences of 92 or more residues reduced the n$_{\text{app}}$ for ACh to a value near that of $\alpha_3\beta_2$ for ACh ($1.1 \pm 0.2$) (Fig. 5 C). Moreover, a single measurement of the n$_{\text{app}}$ (1.1) for $\alpha_3\beta_2(59)$-$\beta_4$ from the initial portion of the log-log dose response relationship suggests that even substituting the first 59 residues of $\beta_2$ for the corresponding region of $\beta_4$ may suffice to reduce the n$_{\text{app}}$ of the chimera to a $\beta_2$-like value. Thus, as for the $\beta_4$ NH$_2$-terminal chimeras, changes in the EC$_{50}$’s of the $\beta_2$ NH$_2$-terminal chimeras were not synchronous with changes in the n$_{\text{app}}$. If the n$_{\text{app}}$ is determined by areas of contact between the subunits, then these results suggest that an extensive region of subunit/subunit contacts, particularly at the NH$_2$-terminals of the $\alpha_3$ and $\beta_4$ subunits, are required for the receptor to display the strong cooperativity observed for the $\alpha_3\beta_4$ receptor. The small n$_{\text{app}}$ of the $\alpha_3\beta_2(120)$-$\beta_4$ raises the possibility that there may also be heterogeneity in the assembly of this receptor. The $\alpha_3\beta_2(92)$-$\beta_4$ and $\alpha_3\beta_2(229)$-$\beta_4$ chimeras also display Hill coefficients that are slightly < 1.

![Figure 6](link)

**Figure 6.** The $\alpha_3\beta_2(229)$-$\beta_4$ chimera displays a $\beta_2$-like insensitivity to 30 $\mu$M Cyt relative to ACh. (A–D) Responses to 30 $\mu$M Cyt and 30 $\mu$M ACh at $-50$ mV. The square pulses above the traces denotes the release of agonist from the U tube.

Exchangeing Just $\beta_2$:104–120 and $\beta_4$:106–122 Shifts the EC$_{50}$ in the Expected Direction

Mutations show that the two $\beta_4$/$\beta_2$ substitutions $\beta_2$:Met101$\rightarrow$Thr103 and $\beta_2$:Phe106$\rightarrow$Val108 are not sufficient to change the EC$_{50}$ of the ACh dose-response relationship. We therefore constructed the reciprocal chimeras $\alpha_3\beta_4$-$\beta_2(104$–120)$-$\beta_4$ and $\alpha_3\beta_2$-$\beta_4(106$–122)$-$\beta_2$ to test whether more extensive substitutions in this area could affect the EC$_{50}$ for ACh. Exchanging $\beta_2$:104–120 and $\beta_4$:106–122 shifted the EC$_{50}$ for ACh towards the opposing wild-type value (Fig. 7, A and B). Substituting $\beta_2$:104–120 for the corresponding region of $\beta_4$ reduced the EC$_{50}$ for ACh to 90 ± 20 $\mu$M ($n = 4$), a value less than half that of $\alpha_3\beta_4$ (219 ± 12 $\mu$M). The reciprocal substitution ($\beta_4$:106–122 for the corresponding region of $\beta_2$) increased the EC$_{50}$ about threefold over the EC$_{50}$ for $\alpha_3\beta_2$, to 28 ± 6 $\mu$M ($n = 8$). Consistent with the results for the $\beta_2$ and $\beta_4$ NH$_2$-terminal chimeras, substituting $\beta_2$:104–120 reduced the cooperativity of the chimera to 1.3 ± 0.1 ($n = 4$) while the reciprocal substitution did not significantly increase the n$_{\text{app}}$ for ACh (1.3 ± 0.2, $n = 8$). Thus, eight residue
substitutions in β2:104–120 and β4:106–122 (see Fig. 1) had an effect on the EC_{50} and n_{app} for ACh that was independent of substitutions elsewhere in the β subunits.

Substituting β2:104–120 for β4:106–122 Also Affects Agonist Selectivity

Previous data (Figl et al., 1992) show that extending the β4 region of a β4 NH₂-terminal chimera from β4:1–105 to β4:1–122 increases the 30 μM CYT/30 μM ACh (CYT/ACh) and 100 μM TMA/30 μM ACh (TMA/ACh) response ratios from β2-like to β4-like levels. We tested the effects of exchanging β2:104–120 and β4:106–122 to determine whether these regions accounted for the effects of the β4 NH₂-terminal chimeras on agonist selectivity.

Substituting β2:104–120 for β4:106–122 reduced the CYT/ACh and TMA/ACh response ratios to β2-like values (Fig. 8, A and B), but the reciprocal substitution β4:106–122 for β2:104–120 produced only a small increase in the CYT/ACh or TMA/ACh response ratios toward β4-like values. Thus, similar to the effects of β2 substitutions on cooperativity, short internal substitutions reduce, but do not restore, the relative sensitivity of the α3β4 receptor to ganglionic agonists such as CYT and TMA. The structural basis for this correspondence between the effects of internal substitutions on agonist selectivity and cooperativity is unclear. Nonetheless, the
effects of exchanging \( \beta_4:106-122 \) and \( \beta_2:104-120 \) on several measurements associated with agonist activation of the receptor suggest that these areas are places where the \( \alpha \) and \( \beta \) subunits contact to form an agonist binding site.

**The Sole M2 Substitution Does Not Affect the ACh Dose-Response Relationship**

Dose-response relationships for nAChRs are affected by mutations at several residues in the M2 transmembrane domain (reviewed in Karlin, 1993). There is a single \( \beta_2/\beta_4 \) substitution in the M2 region (\( \beta_2:Val253\leftrightarrow\beta_4:Phe255 \)). We made the reciprocal point mutations \( \beta_4:\text{Val}_{255} \leftrightarrow Phe_{255} \) and \( \beta_2:\text{Phe}_{255} \leftrightarrow Val_{255} \) to test whether this substitution could shift the agonist dose-response relationship. Neither mutation significantly affected the EC\( \text{so} \) or n\( \text{app} \). The n\( \text{app} \) of \( \alpha 3\beta_4\text{Val}_{255} \) was 2.0 ± 0.1 (\( n = 3 \)); the n\( \text{app} \) of \( \alpha 3\beta_2\text{Phe}_{255} \) was 1.2 ± 0.1 (\( n = 3 \)); the EC\( \text{so} \) of \( \alpha 3\beta_4\text{Val}_{255} \) was 231 ± 18 \( \mu \text{M} \) (\( n = 3 \)); and, the EC\( \text{so} \) of \( \alpha 3\beta_2\text{Phe}_{255} \) was 9 ± 1 \( \mu \text{M} \) (\( n = 3 \)).

**DISCUSSION**

The data reported in this paper describe the steady state dose-response relations of chimeric and mutated \( \beta_2 \) and \( \beta_4 \) subunits expressed with the \( \alpha 3 \) subunit in oocytes.
The data show that the first 120 residues of β2 and a comparable, but slightly shorter, region of β4 largely account for the different EC₅₀'s of the wild-type dose-response relationships. In the sharpest region of transition, chimeras that differ at only two residues have more than a 10-fold difference in the EC₅₀. However, when we examined the corresponding mutant β²(M101T,F106V) it retained the EC₅₀ of the wild-type β2 subunit. This important result shows that the two critical residues are interacting with other upstream residues during activation of the receptor. Longer substitutions in this region (β2:104-120→β4:106-122) do affect (a) the ACh dose-response relationship and (b) the relative responses to several ganglionic agonists.

The domains we have identified that affect the EC₅₀ probably affect agonist binding and may contribute to an agonist binding site that bridges the α and β subunits.

Our experiments also bear on the regions responsible for the cooperativity of the receptor (nₐₚₚ). Regions of the β subunits responsible for differences in the nₐₚₚ overlap with, but are not identical to, those responsible for differences in the EC₅₀. The β2:104–120 and β4:106–122 regions affect both measures. However, for the β4 NH₂-terminal chimeras, the transition from β2-like to β4-like cooperativity occurs more gradually than the transition from a β2-like to a β4-like EC₅₀ and requires an extensive NH₂-terminal region of the β4 subunit. The high level of cooperativity of αβ4 is disrupted by short β2 substitutions. If receptor cooperativity is determined by areas of contact between the subunits (Koshland et al., 1966), then one of these contact regions appears to lie in β2:104–120 and β4:106–122.

The differences we observed between the ACh dose-response relationships of the αβ2 and αβ4 receptors are similar to those reported previously by Couturier et al. (1990), Leutje et al. (1993), and Wong et al. (1993) but differ substantially from those reported by Cachelin et al. (1991). Cachelin et al. (1991) report a much smaller value for the EC₅₀ of αβ4 for ACh (30 μM) and a much larger value for the EC₅₀ of αβ2 for ACh (354 μM). They report similar, but less diverse, nₐₚₚ's for the two receptor subtypes (nₐₚₚ = 1.4 for αβ2; nₐₚₚ = 1.8 for αβ4). The reason for this discrepancy is unclear but most of the previous measurements, including data from a mammalian expression system (Wong et al., 1993), are consistent with our data.

The αβ2(229)β4 chimera is anomalous because it displays a large, β4-like EC₅₀ for ACh even though it includes regions of sequence that confer a β2-like EC₅₀ in another chimera tested. Nonetheless, the αβ2(229)β4 chimera exhibits a β2-like CYT/ACh response ratio and a β2-like irreversible block by neuronal bungarotoxin (Papke et al., 1993). Based on its agonist selectivity and affinity for neuronal bungarotoxin, we suggest that the large EC₅₀ of this chimera arises from a change in the conformational states of the receptor that enhances the relative stability of the closed singly liganded receptor state (Koshland et al., 1966) rather than from a change in the agonist binding site.

Channel Block by the Agonist Fails to Account for Differences in the Nₐₚₚ

Measurements of the Hill coefficient at the foot of the dose-response relationship show that the differences between the nₐₚₚ's of the wild-type receptors are not due to channel block by the agonist because open-channel block is minimal in this region. However, open-channel block by the agonist could reduce the apparent EC₅₀ of the agonist dose-response relationship. To assess the importance of this effect, we
calculated the dissociation constant $K_i$ of agonist for an open-channel blocking site that would yield a $\beta_2$-like EC$_{50}$, assuming that (a) the agonist can open all the channels (a worst-case analysis), (b) $\alpha_3\beta_2$ and $\alpha_3\beta_4$ display a $\beta_4$-like EC$_{50}$ for ACh (219 $\mu$M) in the absence of channel block, and (c) the $n_{app}$ for $\alpha_3\beta_2$ is $\sim$1. The fractional response of the receptor ($I_{peak}/I_{max}$) under these conditions is

$$\frac{I_{peak}}{I_{max}} = \frac{1}{1 + \frac{219 \, \mu\text{M}}{[\text{ACh}]} + \frac{[\text{ACh}]}{K_i}}$$

A $K_i$ of 10 $\mu$M would be required to bring the EC$_{50}$ to 24 $\mu$M. Concentration- and voltage-jump experiments on neuronal nAChRs in bovine chromaffin cells suggest that the actual dissociation constant of ACh for the channel blocking site of neuronal nAChRs is closer to 700–1,400 $\mu$M (Maconochie and Knight, 1992), or 70- to 140-fold greater than the value required to bring the EC$_{50}$ for $\alpha_3\beta_2$ to 24 $\mu$M. Moreover, with a $K_i$ of 10 $\mu$M, the maximum response would occur at $\sim$50 $\mu$M ACh. Higher agonist concentrations would reduce the amplitude of the response, contrary to our data for $\alpha_3\beta_2$ (Fig. 2A). Additionally, the sole substitution in M2, which is thought to contain the binding site for open-channel blockers, has no effect. These results strongly suggest that agonist self-block does not account for the differences in the EC$_{50}$’s that we observed.

**Residues in a Homologous Region of $\gamma$ and $\delta$ Determine Curare Binding**

Three residue substitutions in the mouse $\gamma$ and $\delta$ subunits ($\gamma$:Ile116$\leftrightarrow$Val118, $\gamma$:Tyr117$\leftrightarrow$Thr119, $\gamma$:Ser161$\leftrightarrow$Lys163) account for a 10-fold increase in the affinity of the $\alpha_3\gamma$ receptor over the $\alpha_3\delta$ receptor for the competitive antagonist (+)-dimethyltubocurarine (Sine, 1993). Residues $\beta_2$:Ile118 and $\beta_2$:Phe119 in the rat neuronal nicotinic receptor are homologous to $\gamma$:Ile116 and $\gamma$:Tyr117 in the mouse receptor and they are in the $\beta_2$:104–120 domain of the rat neuronal nicotinic receptor. More recent evidence (Xie, Chiara, and Cohen, 1994) shows that the competitive antagonist dTC also photolabels residues $\gamma$:Tyr111 and $\gamma$:Tyr117 in the *Torpedo* nAChR (homologous to $\beta_2$:Ser113 and $\beta_2$:Phe119 in the rat neuronal nicotinic receptor). Thus, in a domain homologous to $\beta_2$:104–120 in the rat neuronal nicotinic receptor, we find (a) residues in the *Torpedo* $\gamma$ subunit that are photolabeled by dTC and (b) residues in the mouse $\gamma$ and $\delta$ subunits that affect competitive antagonist binding. Residues in $\beta_2$:104–120 affect the $n_{app}$, the EC$_{50}$, and agonist selectivity of neuronal nicotinic receptors. The proximity of residues in the muscle-type nicotinic receptors that affect binding and photolabeling by a competitive antagonist with those in the neuronal nicotinic receptor that affect the $n_{app}$, the EC$_{50}$, and agonist selectivity supports the hypothesis that residues $\beta_2$:104–120 contribute to an $\alpha/\beta$ ligand binding site in neuronal nAChRs.

**Differences in Channel Gating Do Not Account for Differences in the Wild-Type EC$_{50}$**

We consider a simple sequential model to suggest that differences in agonist binding, rather than in the rates of conformational change, are primarily responsible for the
difference between the EC$_{50}$'s of $\alpha_3\beta_2$ and $\alpha_3\beta_4$ for ACh. This kinetic scheme (Scheme II) can be summarized as follows,

\[
nA + R \xrightleftharpoons[K_1]{K_2} C \xrightarrow[K_3]{} O \xrightarrow[]{} I,
\]

**Scheme II**

where $n$ is the Hill coefficient, $A$ is the agonist, $R$ is the free receptor, $C$ is the closed agonist-bound receptor, $O$ is the conducting state of the receptor, $I$ is an agonist-bound inactivated state of the receptor, and $K_1$--$K_3$ are dissociation constants. At equilibrium, the fraction of open receptors $P_o$ is,

\[
P_o = \frac{1}{1 + \frac{K_2}{K_3} + \frac{K_2}{[A]^n} + K_1^n}.
\]

The EC$_{50}$ is,

\[
EC_{50} = K_1^n \sqrt[3]{\frac{K_2K_3}{1 + K_3 + K_3K_2}}.
\]

According to the data of Papke and Heinemann (1991), $K_2 = 1.1$ and 0.45, and $K_3 = 10$ and 20 for $\alpha_3\beta_2$ and $\alpha_3\beta_4$, respectively. The point here is not the absolute values of these numbers, but the fact that they differ by relatively small amounts for the two receptors. A similar conclusion is reached from our own kinetic data on voltage-jump relaxations, to be published separately. These differences would change the EC$_{50}$ by less than twofold. Thus, differences in the cooperativity of binding and conformational transitions of $\alpha_3\beta_2$ and $\alpha_3\beta_4$ cannot account for the 20-fold difference we observed between the EC$_{50}$'s of the wild-type receptors for ACh. This model suggests that the wild-type dissociation constants for agonist binding ($K_1$'s) differ by more than 10-fold.

Regardless of the particular kinetic model used, the differences between the mean open times of the $\alpha_3\beta_2$ and $\alpha_3\beta_4$ channels do not account for the differences in the EC$_{50}$'s of $\alpha_3\beta_2$ and $\alpha_3\beta_4$ for ACh. The open channel lifetime is usually interpreted as the rate constant for leaving the open state of the channel. The long open-channel lifetime of the primary $\alpha_3\beta_4$ channel is 75% greater than the long open channel lifetime of the primary $\alpha_3\beta_2$ channel (Papke et al., 1991). All else being equal, this difference in open channel lifetime would be associated with a smaller EC$_{50}$ for $\alpha_3\beta_4$ than for $\alpha_3\beta_2$, contrary to the data.

**Conclusions**

Our study is the first attempt to determine the regions of sequence that account for the differences in the complete agonist dose-response relationship produced by two naturally occurring subunits of a channel. Past experiments on binding of antagonists or open-channel blockers have pinpointed just a few residues; however, channel activation in response to agonists is certainly a more complex phenomenon, involving binding, conformational changes, and interactions between the subunits. It
is therefore encouraging that clear trends emerge from the data. Our results emphasize the involvement of one region, β2:104–120, in the binding event and suggest that a broader region of sequence is involved in the subunit contacts and/or conformational changes that open the channel.

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