α4β2 Nicotinic Acetylcholine Receptors

RELATIONSHIPS BETWEEN SUBUNIT STOICHIOMETRY AND FUNCTION AT THE SINGLE CHANNEL LEVEL

Acetylcholine receptors comprising α4 and β2 subunits are the most abundant class of nicotinic acetylcholine receptor in the brain. They contribute to cognition, reward, mood, and nociception and are implicated in a range of neurological disorders. Previous measurements of whole-cell macroscopic currents showed that α4 and β2 subunits assemble in two predominant pentameric stoichiometries, which differ in their sensitivity to agonists, antagonists, and allosteric modulators. Here we compare agonist-elicited single channel currents from receptors assembled with an excess of either the α4 or β2 subunit, forming receptor populations biased toward one or the other stoichiometry, with currents from receptors composed of five concatemeric subunits in which the subunit stoichiometry is predetermined. Our results associate each subunit stoichiometry with a unique single channel conductance, mean open channel lifetime, and sensitivity to the allosteric potentiator 3-[3-(3-pyridinyl)-1,2,4-oxadiazol-5-yl]benzonitrile (NS-9283). Receptors with the composition (α4β2)α4 exhibit high single channel conductance, brief mean open lifetime, and strong potentiation by NS-9283, whereas receptors with the composition (α4β2)β2 exhibit low single channel conductance and long mean open lifetime and are not potentiated by NS-9283. Thus single channel current measurements reveal bases for the distinct functional and pharmacological properties endowed by different stoichiometries of α4 and β2 subunits and establish pentameric concatemers as a means to delineate interactions between subunits that confer these properties.

Nicotinic acetylcholine receptors (AChRs)2 are pentameric ligand-gated ion channels formed from homologous subunits of which there are many different subtypes (1). The ability to combine different types of subunits into an individual penta-
So far, relationships between the stoichiometry of α4 and β2 subunits and the functional properties of a receptor have been inferred from measurements of agonist-elicited macroscopic currents that yield the average response of the overall receptor population. In addition, functional properties such as unitary conductance, open channel lifetime, and kinetics of activation and deactivation are largely inaccessible to macroscopic current recording. Here, using patch clamp electrophysiology, agonist-elicited single channel currents were recorded from receptors formed in the presence of an excess of either the α4 or β2 subunit. These unitary currents were then compared with those from concatemeric receptors in which the stoichiometry and positioning of the subunits is predetermined. The results reveal relationships between the stoichiometry of α4 and β2 subunits and functional properties at the level of single receptor channels.

Results

Identification of Single Channel Currents from α4β2 nAChRs—Patch clamp recordings, before and after addition of agonist, are required to unambiguously demonstrate single channel currents from α4β2 AChRs. Using an agonist-free recording pipette, a gigaohm seal was established to a BOSC 23 cell transfected with equal amounts of unlinked α4 and β2 subunit cDNAs. The baseline current was recorded to verify the absence of single channel currents, and then the membrane-permeable agonist nicotine was added to the extracellular solution (Fig. 1A). After a short delay, single channel openings appeared, initially at a high frequency followed by a slow decline due to desensitization. Inspection of individual channel openings reveals two distinct unitary current amplitudes of 3.2 ± 0.4 and 4.1 ± 0.5 pA, in agreement with previous studies (9, 20). Applying the same procedure to a BOSC cell transfected with cDNA encoding a pentameric concatemer composed of three non-consecutive α4 and two β2 subunits reveals nicotine-activated single channel currents that again decline in frequency due to desensitization (Fig. 1B). Inspection of individual channel openings reveals one unitary current amplitude of 4.3 ± 0.5 pA, in contrast to the dual amplitudes observed with unlinked subunits. These observations establish the identity of agonist-elicited single channel currents from α4β2 AChRs formed by unlinked as well as concatemeric subunits. Furthermore, they suggest unitary current amplitude depends on subunit stoichiometry.

Subunit Stoichiometry and Single Channel Current Amplitude—To establish that the different unitary current amplitudes arise from receptors with different subunit stoichiometry, agonist-elicited single channel currents were recorded following transfection of an excess of either the α4 or β2 subunit cDNA, as opposed to the equal amounts in Fig. 1A. During receptor assembly, an excess of one subunit over the other is expected to bias the receptor population toward pentamers.
Single Channel Properties of α4β2 nAChR

A

Acetylcholine

2 pA

20 ms

B

C

D

E

F

G

TC-2559

with an excess of the surplus subunit. To determine subunit stoichiometry, recordings from receptors composed of unlinked subunits were compared with receptors composed of concatemeric subunits containing either two or three α4 subunits plus the pentameric complement of β2 subunits. For these determinations, single channel currents were recorded in the cell-attached patch configuration with a concentration of 10 μM ACh in the recording pipette and a holding potential of −70 mV. Under these conditions, the concentration of ACh exposed to the receptors is held constant, which establishes an equilibrium between activatable and desensitized receptors. When unlinked α4 and β2 subunits were transfected in a 1:1 ratio, the single channel current traces, as well as a histogram of single channel current amplitudes, again reveal two distinct current amplitudes of 3.4 and 4.2 pA (Fig. 2A); the histogram is well described by the sum of two Gaussian components. However, when α4 and β2 subunits were transfected in a 1:10 ratio, a condition expected to promote assembly of receptors with two rather than three α4 subunits, the single channel traces, as well as the histogram of current amplitudes, reveals one current amplitude of 3.4 pA (Fig. 2B). Conversely, when the α4 and β2 subunits were transfected in a 10:1 ratio, a condition expected to promote assembly of receptors with three rather than two α4 subunits, the single channel current traces and the histogram of current amplitudes reveal one current amplitude of 4.1 pA (Fig. 2C). Thus the unitary current amplitudes obtained from receptors formed with biased ratios of α4 and β2 subunits recapitulate those of receptors formed with an equal ratio of the subunits.

Recordings from concatemeric receptors comprising two non-consecutive α4 subunits and three β2 subunits reveal one current amplitude of 3.4 pA (Fig. 2D), in close accord with recordings from receptors formed using a 1:10 ratio of unlinked α4 and β2 subunits (Fig. 2B). In contrast, recordings from concatemeric receptors comprising three α4 subunits and two non-consecutive β2 subunits reveal one current amplitude of 4.2 pA (Fig. 2E), in close accord with recordings from receptors formed using a 10:1 ratio of unlinked α4 and β2 subunits (Fig. 2C). Thus, based on measurements of unitary current amplitude, the subunit stoichiometries inferred for receptors formed using biased ratios of unlinked subunits coincide with the known subunit stoichiometries in concatemeric receptor pentamers.

To quantify the unitary conductance for receptors of each subunit stoichiometry, single channel currents were recorded at different holding potentials, and histograms of single channel current amplitudes were analyzed (Fig. 2F). For each receptor, a plot of the mean current amplitude against holding potential reveals a straight line, the slope of which gives the unitary conductance. Receptors containing two α4 and three β2 subunits,
Single Channel Properties of $\alpha_4\beta_2$ nACHR

whether formed from a 1:10 ratio of unlinked subunits or a concatemeric receptor composed of two non-consecutive $\alpha_4$ and three $\beta_2$ subunits, exhibit a unitary conductance of 48–49 pS. By contrast, receptors containing three $\alpha_4$ and two $\beta_2$ subunits, whether formed from a 10:1 ratio of unlinked subunits or a concatemeric receptor composed of three $\alpha_4$ and two non-consecutive $\beta_2$ subunits, exhibit a unitary conductance of 60 pS. Thus measurements of single channel current amplitudes associate a defined stoichiometry and positioning of subunits within the pentamer with a distinct unitary channel conductance.

To confirm the association between unitary current amplitude and subunit stoichiometry, single channel currents elicited by the agonist 4-((5-ethoxy-3-pyridinyl)-N-methyl-(3E)-3-buten-1-amine difumarate (TC-2559) were recorded in the cell-attached patch configuration at a holding potential of $-70 \text{ mV}$ (Fig. 2G). TC-2559 was chosen because it is a selective agonist for $\alpha_4\beta_2$ AChRs (21). In all cases, the current amplitude from each concatemeric receptor pentamer concurs with that from the corresponding receptors formed from biased ratios of unlinked $\alpha_4$ and $\beta_2$ subunits. Thus, based on recordings with multiple agonists (nicotine, ACh, and TC-2559), receptors with greatest unitary conductance correspond to pentamers with three $\alpha_4$ and two non-consecutive $\beta_2$ subunits, whereas receptors with lowest unitary conductance correspond to receptors with two non-consecutive $\alpha_4$ and three $\beta_2$ subunits.

Subunit Stoichiometry and Open Channel Lifetime—To determine whether receptors with different subunit stoichiometry differ in their open channel lifetimes, single channel currents elicited by a concentration of 10 $\mu$M ACh were recorded in the cell-attached patch configuration at a holding potential of $-70 \text{ mV}$ (Fig. 3). The concentration of ACh was designed to minimize ACh-mediated channel block while still being sufficient to activate receptors with either high or low sensitivity to ACh. Receptors formed with a 1:10 ratio of unlinked $\alpha_4$ to $\beta_2$ subunits exhibit four exponential components of channel openings with mean durations that differ by approximately 1 order of magnitude (Fig. 3A and Table 1). The mean duration of the briefest component is $\sim 40 \mu$s, whereas that of the longest component is $\sim 10 \text{ ms}$. Analysis of single channel current amplitudes from the same recording reveals one amplitude of $\sim 3 \text{ pA}$, verifying that the stoichiometry of these receptors is two $\alpha_4$ and three $\beta_2$ subunits.

Concatemeric receptors comprising two non-consecutive $\alpha_4$ and three $\beta_2$ subunits also exhibit four exponential components of openings with mean durations similar to those of receptors formed by unlinked subunits (Fig. 3B and Table 1). Previous measurements of macroscopic currents from the same concatemeric receptors reveal an $\text{EC}_{50}$ of $\sim 1 \mu$M (18), suggesting that the ACh concentration of 10 $\mu$M ACh used in the single channel recordings approaches full occupancy of the ligand binding sites.

In contrast, receptors formed with a 10:1 ratio of unlinked $\alpha_4$ to $\beta_2$ subunits exhibit three exponential components of channel openings with mean durations similar to those of the three briefest components by receptors formed with a 1:10 ratio of subunits. Analysis of single channel current amplitudes from the same recording reveals one amplitude of $\sim 4 \text{ pA}$, verifying
that the stoichiometry of these receptors is three α4 and two β2 subunits. Concatemeric receptors comprising three α4 and two non-consecutive β2 subunits also exhibit three exponential components of openings, although the mean durations are somewhat longer than those of the corresponding receptors formed by unlinked subunits. Previous measurements of macroscopic currents from these concatemeric receptors reveal an EC_{50} of ~100 μM, indicating that the ACh concentration of 10 μM in the single channel recordings achieves only partial occupancy of the ligand binding sites. Thus the two different subunit stoichiometries give rise to receptors with distinct distributions of open channel lifetimes in which a component with long mean duration is observed for receptors with two α4 and three β2 subunits, whereas receptors with three α4 and two β2 subunits lack this component. However, this distinction could arise either from differences in the inherent stability of the open channel or from differences in the number of bound agonists.

**Subunit Stoichiometry and Drug Potentiation—**The drug 3-[3-(3-pyridinyl)-1,2,4-oxadiazol-5-yl]benzonitrile (NS-9283) potentiates αβ2 receptors and is selective for receptors in which the number of α4 subunits is greater than the number of β2 subunits. Potentiation arises via a left shift of the agonist concentration-response relationship rather than an increase of the maximum response. Thus measurements of potentiation of α4β2 receptors by NS-9283 were designed to further distinguish between receptors with different subunit stoichiometry (Fig. 4). A gigaseal seal was formed to a cell transfected with a 10:1 ratio of α4 to β2 subunit cDNAs, and ACh was included in the recording pipette without or with NS-9283. The ACh concentration of 10 μM is below the EC_{50} for receptors with three α4 and two β2 subunits, and the NS-9283 concentration of 10 μM approaches that which maximally potentiates macroscopic currents and is below that which produces channel block. In the presence of ACh alone, channel openings appear as random isolated events in the presence of both ACh and NS-9283, histograms comprise three exponential components of channel openings either without or with NS-9283 (Fig. 4, C and D). Open duration histograms comprise three exponential components of channel openings in rapid succession.

To further quantify potentiation by NS-9283, the fraction of channel openings that were followed by a reopening is plotted against the number of reopenings (Fig. 5). A reopening is defined as an opening preceded by a closing shorter than the critical closed time used to define clusters. In the presence of ACh alone, the distribution is well described by a single exponential decay, and the mean number of reopenings, 0.4–0.5, is similar whether the receptors comprise unlinked or linked subunits. In the presence of ACh and NS-9283, a second exponential component is observed corresponding to potentiated receptors. For this second component, the mean number of reopenings increases markedly to 3.5 for receptors formed from unlinked subunits and to 9.9 for receptors formed from linked subunits. Thus potentiation by NS-9283 arises from a marked increase in the fraction of channel openings that are followed by a reopening, as well as an increase in the number of reopenings per activation episode.

Whether NS-9283 potentiation depends on subunit stoichiometry was evaluated by applying the experimental procedures described in Fig. 4 to cells transfected with either a 1:10 ratio of α4 to β2 subunit cDNAs or concatemeric receptor pentamers with two non-consecutive α4 subunits and three β2 subunits. For a recording from receptors formed from unlinked subunits, the open duration histogram comprises four exponential com-
Single Channel Properties of α4β2 nAChR

A
ACH 10 μM

5 pA
50 ms

Closed
Open
Cluster

B
ACH 10 μM + NS-9283 10 μM

C
ACH 10 μM

D
ACH 10 μM + NS-9283 10 μM
TABLE 2
Open durations of unlinked and linked receptors activated by ACh ± NS-9283

Mean open times (O) in units of milliseconds and fractional areas (A) are given along with ±.S.E. for the indicated number of patches, n. In cases in which S.E. is not given, the frequency of channel openings in individual patches was low, and the indicated number of patches was combined prior to fitting.

| | O1 (A1) | O2 (A2) | O3 (A3) | O4 (A4) |
|---|---|---|---|---|
| **Unlinked α4β2:1:10** | | | | |
| ACh (n = 2) | 0.022 (0.42) | 0.38 (0.32) | 1.2 (0.12) | 5.4 (0.06) |
| ACh + NS-9283 (n = 2) | 0.016 ± 0.019 (0.38 ± 0.02) | 0.58 ± 0.009 (0.53 ± 0.2) | 2.4 ± 0.6 (0.27 ± 0.18) | 5.4 (0.08) |
| **Linked (α4β2), β2** | | | | |
| ACh (n = 4) | 0.065 (0.21) | 0.83 (0.48) | 1.1 (0.30) | 22 (0.2) |
| ACh + NS-9283 (n = 3) | 0.035 (0.32) | 0.76 (0.27) | 16.7 (0.12) | 16.7 ± 0.25 (0.18 ± 0.05) |

TABLE 3
Cluster durations of unlinked and linked receptors activated by ACh ± NS-9283

Mean cluster durations (Cd) in units of milliseconds and fractional areas (A) are given along with ±.S.E. for the indicated number of patches, n. In cases in which S.E. is not given, the frequency of channel openings in individual patches was low, and the indicated number of patches was combined prior to fitting.

| | Cd1 (A1) | Cd2 (A2) | Cd3 (A3) | Cd4 (A4) | Cd5 (A5) |
|---|---|---|---|---|---|
| **Unlinked α4β2:1:10** | | | | | |
| ACh (n = 2) | 0.034 (0.38) | 0.38 (0.32) | 1.2 (0.19) | 5.4 (0.08) | 21.7 (0.03) |
| ACh + NS-9283 (n = 2; τoff = 1 ms) | 0.067 ± 0.003 (0.20 ± 0.03) | 0.58 ± 0.009 (0.53 ± 0.2) | 2.4 ± 0.6 (0.27 ± 0.18) | 5.4 (0.08) |
| **Linked (α4β2), β2** | | | | | |
| ACh (n = 4; τoff = 1 ms) | 0.028 (0.20) | 0.33 (0.48) | 1.1 (0.30) | 22 (0.2) |
| ACh + NS-9283 (n = 2; τoff = 1 ms) | 0.038 (0.32) | 0.28 (0.40) | 0.76 (0.27) | 5 (0.01) |
| **Unlinked α4β2:10:1** | | | | | |
| ACh (n = 3; τoff = 1 ms) | 0.048 ± 0.002 (0.31 ± 0.16) | 0.55 ± 0.11 (0.53 ± 0.06) | 2.4 ± 0.3 (0.16 ± 0.07) |
| ACh + NS-9283 (n = 3; τoff = 30 ms) | 0.032 (0.44) | 0.25 ± 0.06 (0.17 ± 0.014) | 1.57 ± 0.09 (0.22 ± 0.05) |
| **Linked (α4β2), α4** | | | | | |
| ACh (n = 5; τoff = 1 ms) | 0.028 (0.17) | 0.37 (0.71) | 2.5 (0.13) |
| ACh + NS-9283 (n = 5; τoff = 10 ms) | 0.036 (0.39) | 0.755 (0.34) | 10 (0.26) |

Single channel data from concatamerically receptor pentamers containing three non-consecutive β2 and two α4 subunits mirror those from receptors formed from biased ratios of unlinked subunits, showing briefer rather than longer channel openings in the presence of NS-9283 (Fig. 6, C and D, and Tables 2 and 3). Thus NS-9283 does not potentiate receptors with two α4 and three β2 subunits and is selective for receptors with three α4 and two β2 subunits.

**Discussion**

Previous work, based on measurements of agonist-elicited macroscopic currents, established that α4 and β2 subunits assemble with variable subunit stoichiometry, producing at least two populations of receptor pentamers (7–9). Evidence included a biphasic agonist concentration-response relationship in which the magnitudes of the high and low affinity components varied, depending on the ratios of either the mRNA or cDNA encoding the two subunits. Furthermore, analyses of purified receptors showed that the molar ratio of α4 to β2 subunits depended on the ratio of the mRNAs encoding the two subunits in the presence of ACh alone, whereas the accompanying closed duration histogram contains two brief and two long duration components (Fig. 6A and Table 2). The two brief closed time components represent infrequent reopening of the same receptor channel, generating a minicluster, and the intersection of the second brief with the succeeding long closed time component allows definition of these clusters. The histogram of cluster durations comprises five components, four of which have mean durations similar to those of the open duration histogram, thus representing distinct kinetic classes of isolated channel openings, whereas the fifth component represents successive openings of the same channel (Table 3). For a recording obtained in the presence of both ACh and NS-9283, the open duration histogram comprises four exponential components, and the accompanying closed duration histogram contains two brief and two long duration components (Fig. 6B and Table 2).

The cluster duration histogram comprises three exponential components, the means of which are briefer, rather than longer, than those in the absence of NS-9283, indicating lack of potentiation by NS-9283 and possibly block of the channel (Table 3).
Single Channel Properties of α4β2 nAChR

FIGURE 5. Increase in the number of channel reopenings per cluster by NS-9283 for receptors with three α4 and two β2 subunits. BOSC 23 cells were transfected with the indicated unlinked (A) or linked (B) subunit cDNAs as in Fig. 4. The fraction of clusters with more than a given number of channel reopenings was determined from three to five recordings for each condition and plotted along with S.E. (error bars). For recordings in the presence of ACh alone, a single exponential is fitted to the distributions. For unlinked subunits, the mean number of reopenings per cluster is $\tau = 0.55 \pm 0.007$, whereas that for linked subunits is $\tau = 0.43 \pm 0.04$. For recordings in the presence of ACh and NS-9283, the sum of two exponentials is fitted to the distributions. For unlinked subunits, the mean number of reopenings per cluster and fractional area are $t_1 = 1.16 \pm 0.07, a_1 = 0.68 \pm 0.055$ and $t_2 = 3.5 \pm 0.29, a_2 = 0.32$, whereas for linked subunits, $t_1 = 1.12 \pm 0.13, a_1 = 0.73 \pm 0.04$ and $t_2 = 9.94 \pm 1.6, a_2 = 0.27$.

subunits (9). Studies of pentamic concatemers, in which the stoichiometry and positioning of subunits is predetermined, confirmed that agonist sensitivity depended on the stoichiometric ratio of the α4 and β2 subunits and identified two constructs that recapitulated the high and low agonist sensitivities of receptors formed from unlinked subunits (15, 18). The present results, based on agonist-elicted single channel currents, establish that receptors with different subunit stoichiometry differ in their unitary current amplitudes, kinetics of channel closing, and susceptibility to drug potentiation, as summarized in Table 4. The overall results provide evidence for independent as well interdependent subunit contributions to receptor function and pharmacology.

Previous single channel recordings from receptors comprising α4 and β2 subunits showed multiple unitary current amplitudes, in accord with multiple receptor populations (9, 22, 23). Recently, a study of receptors assembled from biased ratios of unlinked subunits revealed either uniformly high or low unitary conductance, depending on which subunit was present in excess (20). However, the question of subunit stoichiometry remained. Herein, by comparing receptors formed from biased ratios of unlinked subunits with receptors formed from concatemeric subunits, we associate a defined subunit stoichiometry and positioning with a particular unitary conductance; receptors with two non-consecutive α4 and three β2 subunits exhibit a conductance of 48 pS, whereas receptors with two non-consecutive β2 and three α4 subunits exhibit a conductance of 60 pS (Table 4). The conductance differences likely originate from differences in charged residues in the extracellular ring that borders the second transmembrane domain (M2) where the β2 subunit contains a lysine residue and the α4 subunit contains a glutamate (24). The recent crystal structure of the human (α4β2)2 receptor revealed that these residues project into the path of ion flow at the entrance to the pore domain (31). Previous studies showed that these two residues confer differences in calcium permeability between receptors with different stoichiometric ratios of α4 and β2 subunits (12). However, the present studies were done in the absence of calcium, so the conductance difference arises from differences in permeability of monovalent alkali ions. The residues in the inner and intermediate rings of charge, between M1 and M2 at the cytoplasmic side of the membrane, are negatively charged in both the α4 and β2 subunits (24). Thus, as concluded in classical work that unveiled three rings of charge that contribute to ion conductance, the α4 and β2 subunits likely contribute independently of each other according to the sign of the charge in their extracellular ring (25).

Our studies show that receptors with different stoichiometric compositions of subunits differ in their distributions of open channel durations. This observation is significant because each type of receptor contains two ligand binding sites formed at interfaces between α4 and β2 subunits, and occupancy of these sites is coupled to channel opening. Furthermore, an ACh concentration of 10 μM is sufficient to occupy both binding sites on receptors in which the fifth subunit is β2, as these receptors are highly sensitive to ACh. However, when the fifth subunit is α4, a third ligand binding site forms at the interface between α4 and α4 subunits, and the resulting receptors exhibit relatively brief channel openings as shown by the absence of an exponential component of openings with long mean duration. Thus the presence of an α4-α4 ligand binding site has an apparent allosteric effect on the α4-β2 ligand binding sites, either reducing their affinity for ACh or reducing the ability of the channel to open in response to occupancy by ACh. This allosteric effect could originate from a change in symmetry of the α4-β2 binding sites. In particular, for high sensitivity, low conductance receptors, the subunits that form one α4-β2 site are flanked by β2 and α4 subunits, whereas those that form the other site are flanked by β2 and β2 subunits (Table 4). Conversely, for low sensitivity, high conductance receptors, the subunits that form one α4-β2 site are flanked by β2 and α4 subunits, whereas those that form the other site are flanked by α4 and α4 subunits.

Of further importance is our observation that receptors with both stoichiometric compositions of subunits exhibit multiple exponential components of channel openings: four components for high sensitivity, low conductance receptors and three
FIGURE 6. **NS-9283 does not potentiate low conductance receptors with two α4 and three β2 subunits.** BOSC 23 cells were transfected with the indicated unlinked (A and B) or linked (C and D) subunit cDNAs. Single channel currents were recorded in the cell-attached patch configuration with a concentration of 10 μM ACh in the pipette, without or with 10 μM NS-9283, and with a holding potential of −70 mV as in Fig. 4. For each panel, closed, open, and cluster duration histograms fitted by sums of exponentials are shown. The τ_{crit} for generating cluster duration histograms is indicated by the arrow. Fitted parameters are listed in Tables 2 and 3.
components for low sensitivity, high conductance receptors. Assuming that the α4-β2 binding sites are non-equivalent, because of asymmetry in their neighboring subunits, for high sensitivity, low conductance receptors, three components could arise from two species of singly occupied receptors and one component could arise from the doubly occupied receptor (Table 4). However, four components are observed, suggesting that openings may arise from a closed state intermediate between the resting and open channel states (26, 27). For low sensitivity, high conductance receptors, the three observed exponential components could arise from three species of singly occupied receptors, which is plausible given that the agonist concentration is low. With higher concentrations of agonist, additional exponential components are expected due to doubly and triply occupied receptors. Further insights into kinetic mechanisms could thus emerge from studies of the distribution of channel open times as a function of agonist concentration.

Previous studies demonstrated that the drug NS-9283 potentiates receptors with an excess of α4 over β2 subunits but not receptors with an excess of β2 over α4 subunits (11). Our findings confirm that potentiation depends on subunit stoichiometry, but moreover they define the stoichiometry and positioning of subunits that confer potentiation, and reveal the kinetic basis for potentiation. In the absence of NS-9283, individual channel openings appear predominantly in isolation, and the number of reopenings per activation episode follows a single exponential decay. However, in the presence of the drug, a second exponential component is observed comprising clusters of closely spaced events. This change in kinetics is reminiscent of the clustering of channel openings that occurs as the concentration of agonist is increased (28). Thus, although NS-9283 does not elicit channel opening by itself, when it is present together with a low concentration of agonist, the kinetics of channel opening resemble those at high concentrations of agonist. It is possible that NS-9283 acts as a co-agonist of ACh by binding to the α4-α4 binding site, whereas ACh binds to the two α4-β2 sites (29). However, the site for NS-9283 binding could be elsewhere, such as the β2-α4 interface, which does not bind agonist. In this scenario, agonist would bind to three sites, two α4-β2 and the α4-α4, whereas the drug binds to one or both β2-α4 interfaces. Alternatively, NS-9283 may bind at a non-subunit interface, whereas the agonist binds to the three ligand binding sites. Future studies with concatenated receptors could potentially distinguish between these mechanisms.

Our findings not only identify three functional attributes of α4β2 receptors that depend on subunit stoichiometry, but they also identify a positioning of subunits within the pentamer that confers these attributes (Table 4). In particular, in both stoichiometric arrangements, one pair of α4 subunits is separated by a β2 subunit as opposed to being consecutive. This non-consecutive arrangement of α4 subunits is reminiscent of the Torpedo AChR in which the two α1 subunits are non-consecutive (30), and is identical to that in the crystal structure of the (α4β2)β2 receptor (31).

Our findings establish concatenated pentamers combined with single channel measurements as a means to clarify mechanisms of activation and potentiation of α4β2 receptors. By subunit-selective mutagenesis, one could determine whether agonist occupancy of the α4-α4 site alone is responsible for the kinetic differences between receptors with the two stoichiometric compositions of subunits or whether the presence of a third α4 subunit is responsible. Analogously, one could determine whether potentiation by NS-9283 is mediated by a co-agonist mechanism in which the drug binds to the α4-α4 site or whether potentiation arises through binding to non-canonical β2-α4 sites or a site distinct from subunit interfaces. Both investigations require single channel measurements as a functional end point. Finally, our findings offer a means to clarify mechanisms of disease-causing mutations of α4β2 receptors by determining whether the functional consequences of the mutations depend on subunit stoichiometry.

### Experimental Procedures

**Expression of Unlinked and Linked Human α4β2 nAChRs—** cDNAs encoding unlinked α4 and β2 subunits, (α4β2)α4 and (α4β2)β2 concatemeric subunits, and the chaperone protein 14-3-3 were individually subcloned into a modified pCI mammalian expression vector (Promega), as described previously (15, 18). The 14-3-3 chaperone increases expression of α4β2 nAChRs formed by unlinked as well as concatemeric subunits (18, 32). BOSC 23 cells, a cell line derived from HEK 293 cells (33), were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) with 10% fetal bovine serum and transfected by calcium phosphate precipitation, as described previously (34–36). For experiments with receptors formed from unlinked subunits, three different ratios of α4, β2, and 14-3-3 cDNAs were used: 1:1:1, 10:1:1, and 1:10:1. Varying the ratio of α4 to β2 subunit cDNAs biases the receptor population toward a single subunit stoichiometry in both mammalian cell lines (9) and *Xenopus laevis* oocytes (8). The amounts of α4 and β2 cDNAs ranged from 0.3 to 3 μg for each 35-mm culture dish of cells. Transfections were carried out for 4–16 h followed by medium exchange. Single channel recordings were made 48–72 h post-transfection. For experiments with receptors formed from concatenated subunits, (α4β2)α4 and (α4β2)β2, the total amount of concatemer cDNA was 1–10 μg/35-mm culture dish. Furthermore, following transfection at 37°C, the cells were incubated at 30°C until use, which increases expression of receptors on the cell surface (37). Single channel recordings
from cells expressing concatenated receptors were made 72–96 h post-transfection.

Drugs—ACh was purchased from Sigma-Aldrich. TC-2559 and NS-9283 were purchased from Tocris (UK).

Patch Clamp Recordings—Single channel recordings were obtained in the cell-attached patch configuration (38) at a membrane potential of −70 mV and a temperature of 20 °C as described previously (39, 40). For all experiments, the extracellular bathing solution contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl2, 1.7 mM MgCl2, and 10 mM HEPES adjusted to pH 7.4 with NaOH. The pipette solution contained 80 mM KF, 20 mM KCl, 40 mM potassium aspartate, 2 mM MgCl2, 1 mM EGTA, and 10 mM HEPES adjusted to pH 7.4 with KOH (41, 42). Concentrated stock solutions of ACh and TC-2559 were made in DMSO, stored at 80 °C, and added to the pipette solution and stored at −80 °C until the day of each experiment. A concentrated stock solution of NS-9283 was prepared in DMSO, stored at −80 °C, and added to the pipette solution the day of each experiment. Pipette solution without NS-9283 contained an equivalent volume of DMSO to achieve a final concentration of 0.2% (v/v). Patch pipettes were pulled from glass capillary tubes (number 7052, Garner Glass) and coated with Sylgard (Dow Corning).

For experiments in which nicotine was applied to the extracellular bathing solution, a gigaohm seal was initially established to a cell using a patch pipette filled with agonist-free pipette solution. A baseline current was recorded for 1–5 min, and then nicotine was added dropwise to the extracellular solution to establish a final extracellular concentration of 342.5 μM. Continuous recordings were obtained before, during, and after nicotine addition.

Data Analysis—Single channel currents were recorded using an Axopatch 200B patch clamp amplifier (Molecular Devices) with the gain set at 100 mV/pA and the internal Bessel filter at 10 kHz. Currents were sampled at intervals of 20 μs with a PCI-6111E acquisition card (National Instruments) and recorded to hard disk using the program Acquire (Bruxton Corp.). Channel opening and closing transitions were determined using the program TAC 4.2.0 (Bruxton Corp.), which digitally filters the data (Gaussian response; final effective bandwidth, 5 kHz), interpolates the digitized points using a cubic spline function, and detects events using the half-amplitude threshold criterion, as described previously (43). To determine single channel current amplitudes, the variable amplitude option in TAC was used, whereas to determine open and closed dwell times, the fixed amplitude option was used. Dwell time histograms were plotted using a logarithmic abscissa and square root ordinate (44), with a uniformly imposed dead time of 40 μs, and fitted by the sum of exponentials by maximum likelihood using the program TACFiT 4.2.0 (43). Clusters of channel openings were identified as a series of closely spaced openings preceded and followed by closed intervals longer than a specified critical time (τcrit). This duration was taken as the point of intersection between consecutive components in the closed time histogram and ranged between 1 and 30 ms. Cluster duration therefore comprises the total time of a series of openings plus that of the intervening closings briefer than τcrit.

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Single Channel Properties of $\alpha 4\beta 2$ nAChR

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