Non-equivalent Ligand Selectivity of Agonist Sites in \((\alpha 4\beta 2)_2\alpha 4\) Nicotinic Acetylcholine Receptors

A KEY DETERMINANT OF AGONIST EFFICACY*

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Background: The \(\alpha 4\beta 2\) and \(\alpha 4\alpha 4\) interfaces of the \((\alpha 4\beta 2)_2\alpha 4\) nicotinic acetylcholine receptor house structurally different agonist sites.

Results: Agonists of a certain size cannot bind the \(\alpha 4\alpha 4\) interface, which decreases efficacy.

Conclusion: The ability to bind all agonist sites in \((\alpha 4\beta 2)_2\alpha 4\) receptors critically influences agonist efficacy.

Significance: The finding adds a new level of complexity to structural mechanisms governing agonist efficacy.

The \(\alpha 4\beta 2\) nicotinic acetylcholine receptor (nAChR) is the most abundant nAChR type in the brain, and this receptor type exists in alternate \((\alpha 4\beta 2)_2\alpha 4\) and \((\alpha 4\beta 2)_2\beta 2\) forms, which are activated by agonists with strikingly differing efficacies. Recent breakthroughs have identified an additional operational agonist binding site in the \((\alpha 4\beta 2)_2\alpha 4\) nAChR that is responsible for the signature sensitivity of this receptor to activation by agonists, yet the structural mechanisms determining agonist efficacy at this receptor type are not yet fully understood. In this study, we characterized the ligand selectivity of the individual agonist sites of the \((\alpha 4\beta 2)_2\alpha 4\) nAChR to determine whether differences in agonist selectivity influence agonist efficacy. Applying the substituted cysteine accessibility method to individual agonist sites in concatenated \((\alpha 4\beta 2)_2\alpha 4\) receptors, we determined the agonist selectivity of the agonist sites of the \((\alpha 4\beta 2)_2\alpha 4\) receptor. We show that (a) accessibility of substituted cysteines to covalent modification by methanesulfonate reagent depends on the agonist site at which the modification occurs and (b) that agonists such as sazetidine-A and TC-2559 are excluded from the site at the \(\alpha 4\alpha 4\) interface. Given that additional binding to the agonist site in the \(\alpha 4\alpha 4\) interface increases acetylcholine efficacy and that agonists excluded from the agonist site at the \(\alpha 4\alpha 4\) interface behave as partial agonists, we conclude that the ability to engage all agonist sites in \((\alpha 4\beta 2)_2\alpha 4\) nAChRs is a key determinant of agonist efficacy. The findings add another level of complexity to the structural mechanisms that govern agonist efficacy in heteromeric nAChRs and related ligand-gated ion channels.

A central aim in nicotinic acetylcholine receptor (nAChR) physiology and pharmacology is to understand the interactions between agonists and the agonist sites that influence gating efficacy. Functional studies of nAChRs have shown the importance of interactions between agonists and residues on the principal subunit for agonist efficacy (1, 2), and crystal structures of the homolog acetylcholine-binding protein in complex with partial agonists of nAChRs have suggested that partial capping of loop C upon the binding of partial agonists in comparison with the complete capping induced by full agonists is a key determinant of agonist efficacy (3–6). However, acetylcholine-binding protein crystal structures in complex with agonists of nAChRs also show that partial agonists establish water-mediated hydrogen bonds between their hydrogen bond acceptor moiety and the backbone atoms of hydrophobic residues on the complementary face (6, 7), which could affect gating efficacy. Studies of the interactions between agonists and \(\alpha 4\beta 2\) nAChRs by unnatural amino acid mutagenesis have confirmed hydrogen bonds between the complementary face and the hydrogen bond acceptor moieties of partial agonists, although not necessarily mediated by a water molecule (8, 9). Further support for a role of complementary residues on agonist efficacy comes from structural and mutational studies that show that several residues contributed by the complementary subunit affect the efficacy of channel gating (7, 10). Given that agonist sites in heteromeric nAChRs may have different complementary subunits and, hence, different structural elements, the impact of the complementary face on agonist efficacy raises the following fundamental question. Does non-equivalency of agonist sites influence agonist efficacy?

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‡ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; ASA, accessible surface area; DH2E, dihydro-beta-erythroidine; MTS, methanethiosulfonate; MCL, maximum compound length; MTSET, (2-(trimethylammonium)ethyl) methanethiosulfonate; Saz-A, sazetidine-A; TC-2559, 4-(5-ethoxy-3-pyridinyl)-N-methyl-(3E)-3-buten-1-amine difumarate; Var, varenicline; Cyt, cytisine; CI, confidence interval.
Agonist Efficacy and Non-equivalence of Agonist Sites

An ideal nAChR to investigate the role of functional non-equivalency of agonist sites on agonist efficacy is the (α4β2)2α4 nAChR because this protein possesses two types of agonist sites, which are functionally and structurally non-equivalent (11, 12), and because a fully concatenated version of the receptor that aids the study of individual subunit interfaces is available (13). The (α4β2)2α4 nAChR is one of two alternate forms of the α4β2 nAChR (14), the most abundant nAChR type in the brain and a key mediator of the rewarding and reinforcing effects of nicotine (15, 16). Despite having structurally identical agonist binding sites at their α4/β2 interfaces, the alternate receptors display strikingly different sensitivities to activation by agonists and to high-affinity desensitization (14, 17). These differences are accounted for partly by an additional operational agonist binding site at the signature face of the (α4β2)2α4 nAChR (11, 12, 17). From studies of the ACh sensitivity of (α4β2)2α4 nAChRs with an impaired or ablated α4/α4 agonist site (11, 17), it appears that binding of ACh (submaximal concentrations) to the agonist sites at the α4/β2 interfaces produces efficacious gating but that occupation of all three agonist sites (at high concentrations) is necessary for maximal receptor activation. Hence, a likely functional consequence of agonist exclusion from the site at the α4/α4 interface is partial agonism.

Here, we have applied the substituted cysteine accessibility method to concatenated (α4β2)2α4 receptors to address for the first time the agonist selectivity of individual agonist sites in this receptor type. We show that the site at the α4/α4 interface does not accommodate agonists larger than the smoking cessation drug varenicline and that agonists excluded from this site behave as partial agonists. Thus, the ability to engage all agonist sites influences agonist efficacy in (α4β2)2α4 nAChRs and possibly other related neurotransmitter gated ion channels with a (αβ)2α subunit stoichiometry (e.g., the (α3β4)2β4 nAChR (18) and heteromeric glycine receptor (19)).

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression in Oocytes—Xenopus laevis oocytes were prepared from whole ovary tissue obtained from the European Xenopus Resource Center (Portsmouth, UK). Human cRNAs encoding wild type or mutant concatenated (α4β2)2α4 nAChRs were injected into freshly isolated Xenopus oocytes as described previously (11, 13). The fully concatenated form of the (α4β2)2α4 nAChR was engineered as described previously (11, 13). Briefly, the signal peptide and start codon were removed from all of the subunits but the first (a β2 subunit), and the subunits were bridged by AGS linkers. Only the last subunit in the construct (a α4 subunit) contained a stop codon. The subunits were subcloned into a modified pCI plasmid vector (Promega) using unique restriction enzyme sites flanking the N and C termini of each subunit. To introduce a mutation into a specific subunit of the concatemeric (α4β2)2α4 nAChR, the mutation was first introduced into the subunit subcloned into the modified pCI plasmid using the QuikChange site-directed mutagenesis kit (Stratagene). The mutated subunit was then ligated into the concatemer using unique restriction enzyme sites. To confirm that the mutated subunit was incorporated into the concatemer, the subunit was cut from the concatemer using unique restriction enzyme sites, and then its nucleotide sequence was verified by DNA sequencing (Source Bioscience). For clarity, mutations in the concatemeric receptors are shown as superscript positioned in the (+)- or (−)-face of the mutated subunit. Thus, in β2L146C_α4 β2_α4_α4 receptors, the mutation L146C is located in the (−)-face of the β2 subunit occupying the first position of the linear sequence of the concatemer, whereas in β2_α4 β2_α4T152C_α4 receptors, α4T152C is in the (+)-face of the α4 subunit in the fourth position of the linear sequence of the concatemer. All concatemeric constructs were assayed for integrity using restriction enzyme digestion and the LT reporter mutation (L9’T in the second transmembrane domain) as described previously (11).

Oocyte Electrophysiology—Two-electrode voltage clamp recordings on oocytes were carried out as described previously (11, 13). Concentration-response curves for agonists were obtained as described previously (11). Concentration response curves were plotted using Prism version 5.0 (GraphPad Software, San Diego, CA). The agonist activation concentration response curve data were first fit to the one-component Hill equation, \[ I = I_{\text{max}}/\left(1 + (EC_{50}/x)^nH \right), \] where \( EC_{50} \) represents the concentration of agonist inducing 50% of the maximal response \( I_{\text{max}} \), \( x \) is the agonist concentration, and \( nH \) is the Hill coefficient. When agonists induced biphasic receptor activation, the concentration-response curve data were fit to the sum of two Hill equations, as described previously (14).

Methanethiosulfonate (MTS) Modification of Substituted Cysteines—2-Trimethylammonium ethyl methanethiosulfonate (MTSET) (Toronto Research Chemicals, Toronto, Ontario, Canada) was used to covalently modify the introduced free cysteines (20). The accessibility of the introduced cysteines to MTSET was determined using the following protocol. Current responses elicited by 5-s pulses of ACh were measured every 5 min from oocytes expressing wild type or mutant receptors until the ACh responses varied by less than 5% for four consecutive pulses (stabilized responses). For β2_α4 β2_α4T152C_α4 and β2_α4 β2_α4T152C.H142A_α4 receptors, which exhibited monophasic sensitivity to ACh (Figs. 1C and 6, A and B), the concentrations of ACh pulses were 1 and 1.15 mM, respectively. These concentrations corresponded approximately to \( EC_{50} \times 5 \) (see Table 1). For β2L146C _α4 β2_α4_α4 receptors, which exhibited biphasic sensitivity to ACh (see Fig. 1D), the concentration of ACh pulses was \( EC_{50} \times 2 \) (1.6 mM) (Table 1). We have previously reported that impairment of any of the agonist sites in β2_α4 β2_α4_α4 receptors may produce biphasic ACh-induced receptor activation due to the presence of mutated and wild type agonist sites in the concatamer (11). The biphasic curves comprise a high sensitivity component that is contributed mainly by the unaltered agonist sites and a low sensitivity component that is contributed by the mutated and non-mutated agonist site (11). Higher concentrations of ACh (i.e, \( EC_{50} \times 5 \)) were not used on β2L146C _α4 β2_α4_α4 receptors to minimize possible ion channel blockade by ACh and/or chronic receptor desensitization. After stabilization, the MTSET reagent (1 mM) was bath-applied for 120 s, followed by a 90-s wash with Ringer’s solution. Prelimi-
nary experiments showed that this concentration and time of MTSET exposure were sufficient to reach maximal inhibition of the ACh-evoked responses in all mutant receptors tested. After washing, ACh was applied again every 5 min until the amplitude of the responses was constant. The average of the current amplitudes prior to application of MTSET was the control response current ($I_{\text{initial}}$), and the average of current amplitudes after rinsing was the average response after MTSET application ($I_{\text{after-MTSET}}$). The effect of MTSET reagents was estimated using the equation, % Change = ($I_{\text{after-MTSET}}/I_{\text{initial}} - 1) \times 100$.

**Rate of MTSET Modification**—The rate of covalent modification of substituted cysteines by MTSET was determined by measuring the effect of sequential applications of subsaturating concentrations of MTSET using a protocol based on one previously used on GABA$_A$ receptors (21). The concentration of MTSET causing subsaturating effects was determined separately for each mutant receptor. These concentrations were 1 $\mu$M for $\beta_2\omega_4$-$\beta_2\omega_4^{152C\_\omega_4}$ and $\beta_2\omega_4\beta_2\omega_4^{152C\_H142A\_\omega_4}$ receptors and 10 $\mu$M for $\beta_2\omega_4\beta_2\omega_4\alpha_4$ receptors. The responses to ACh prior to MTSET reagent application were first stabilized as follows. $EC_{-50} \times 5$ or $EC_{50}$, depending on the receptor under study, was applied for 5 s, followed by a recovery time of 70 s. Immediately after the recovery time, a pulse of a ligand to be tested later for protection (e.g. ACh, cytisine, varenicline, TC-2559, or sazetidine-A) was applied for 10 s, followed by a 3 min and 40-s wash with Ringer solution. This cycle was repeated until the ACh responses stabilized (<5% variance of peak current responses to ACh on four consecutive applications) (Fig. 3A). Ligands to be tested for their ability to protect the introduced cysteine residues from MTSET reactions (i.e. protectants) were applied during the stabilization of the ACh responses to correct for any process of desensitization and/or ion channel blockade that could develop during the protection assays described below. MTSET was then applied using the following sequence of reactions. At time 0, ACh was applied for 5 s, followed by a period of recovery of 70 s; MTSET was then applied for 10 s, followed by a recovery period of 20 s (Fig. 3A). Immediately after the recovery time, the protectant was applied for 10 s, after which time the cell was washed with Ringer’s solution for 3 min and 40 s. This cycle was repeated until the peak current responses to ACh no longer changed, indicating completion of the MTSET reaction. After completion of the MTSET reaction, ACh and protectant were applied as described above to demonstrate that the observed changes in ACh responses were induced by MTSET (Fig. 3A).

**Protection Assays with Ligands**—To determine whether the accessibility of the incorporated cysteines could be altered by the presence of agonists or antagonist, the following protocol was used. Peak current responses to 5-s pulses of ACh ($EC_{-50} \times 5$ or $EC_{50-2}$) were stabilized as described above, after which time MTSET was applied using the following sequence. At time 0, ACh was applied (5 s), followed by 70-s recovery; MTSET and the protectant for agonists or $IC_{50}$-s for the antagonist DhβE were then co-applied for 10 s, followed by a recovery period of 3 min and 40 s (Fig. 3B). This cycle was repeated nine times (90 s in total). At the end of this cycle, ACh and protectant were applied as described for the MTSET reaction rate protocol. The concentrations of ligands used during the stabilization of the control ACh responses and for the protection assays were as follows: for $\beta_2\alpha_4\beta_2\alpha_4^{152C\_\alpha_4}$, 1 $\mu$M MTSET, 1 mM ACh, 70 $\mu$M cytisine, 12 $\mu$M varenicline, 12 $\mu$M TC-2559, 1 $\mu$M sazetidine-A; for $\beta_2\alpha_4\alpha_4\beta_2\alpha_4\alpha_4$, 10 $\mu$M MTSET, 1.6 mM ACh, 44 $\mu$M varenicline, 65 $\mu$M TC-2559, 1 $\mu$M sazetidine-A. For cytisine protection assays on $\beta_2\alpha_4\alpha_4\beta_2\alpha_4\alpha_4$ receptors, the concentration of cytisine used was 0.3 $\mu$M, which corresponds approximately to an $EC_{50}$. Preliminary studies showed that higher concentrations of cytisine slowed the MTSET reaction to the extent that it was not possible to measure the rate over the time course of the experiments. At lower concentrations (1–10 nM), cytisine did not protect the substituted cysteine. At the end of each protection assay, the cells were exposed to maximal MTSET to ensure that the previously protected mutant cysteines were still accessible. To study the effects of MTSET on $\beta_2\alpha_4\beta_2\alpha_4^{152C\_H142A\_\omega_4}$ receptors, the ACh pulses were 1.15 $\mu$M ($EC_{50} \times 5$; see Table 1), whereas the protectants tested, TC-2559 and sazetidine-A, were used at 20 $\mu$M (approximately $EC_{50} \times 5$). Preliminary experiments indicated that at these concentrations, TC-2559 and sazetidine-A permitted accurate measurements of the rate of MTSET reaction.

For all rate experiments, the decrease in the peak current response to ACh was plotted versus cumulative time of MTSET exposure. The change in response to ACh after cumulative time of MTSET addition ($t$) was expressed relative to the response to ACh prior to the MTSET addition, at $t = 0$. The data expressed in this way were fit with a single exponential decay curve to obtain an estimate of the first order rate constant ($k$) and final current response ($I$) according to the equation, $I = I_\infty + (1 - I_\infty)e^{-k_1t}$. A second-order rate constant ($k_2$) was calculated by dividing $k_1$ by the concentration of MTSET used (21).

**Structure Homology Modeling and Docking**—Sequences of the human α4 and β2 nAChR subunits were obtained from the ExPASy proteomics server (22) with accession numbers P43681 (α4) and P17787 (β2). The homopentameric *Lymanea stagnalis* acetylcholine-binding protein structure (Protein Data Bank code 1UW6) (23) was used to generate models of the extracellular domain of the α4β2 complex. Molecular docking of nicotinergic ligands at the agonist binding sites of the α4β2 homology models was investigated using the Lamarckian genetic algorithm search method as implemented in AutoDock version 4.0, as described previously (24). Statistical Analysis—Data analysis was carried out using non-linear regression analysis included in the Prism software package (GraphPad Software). An F-test determined whether the one-site or biphasic model best fit the concentration response data; the simpler one-component model was preferred unless the extra sum-of-squares F-test had a value of $p$ less than 0.05. Statistical analysis on agonist efficacies or on MTSET accessibility was conducted using a one-way analysis of variance, followed by a post hoc Dunnett’s or Bonferroni test. Statistical differences between control and test log $EC_{50}$ values were determined using unpaired Student’s $t$ tests.
**Agonist Efficacy and Non-equivalence of Agonist Sites**

To study the agonists at the agonist site at the α4/β2 interface, we incorporated B2L146C into the first position of the β2_α4_β2_α4_α4 (α4/β2-1 interface) and produced a β2L146C-α4_β2_α4_α4_α4 mutant receptor (Fig. 1B). The agonist site at the α4/β2 interface adjacent to the fifth subunit of the concatamer (α4/β2-2 interface) was not studied because we found previously that the agonist sites at α4/β2-1 and α4/β2-2 interfaces respond similarly to alanine or cysteine substitutions (11), suggesting functional equivalency of these sites.

Wild type and mutant receptors, expressed heterologously in *Xenopus* oocytes, were activated in a concentration-dependent manner in response to ACh (Fig. 1, C and D). For β2_α4_β2_α4_α4_α4 receptors, the concentration-response curve for ACh was monophasic and shifted to the right by 2.4-fold, compared with wild type (Fig. 1C and Table 1). For β2L146C_α4_β2_α4_α4 receptors, the ACh concentration-response curve was biphasic (p < 0.001; n = 6), comprising a high- and a low-sensitivity component (Fig. 1D and Table 1). Introduction of single point mutants into individual agonist sites of concatenated (α4β2), α4 nAChRs may produce biphasic ACh concentration-response curves, depending on the extent to which mutations decrease the sensitivity of the mutated agonist site to ACh (11). The high-sensitivity component of the curve is contributed mostly by intact agonist sites, whereas the low-sensitivity component is contributed by both intact and impaired agonist sites (11). Mutant receptors were also responsive to partial agonists, varenicline, TC-2559, and sazetidine-A (Table 1). For β2L146C_α4_β2_α4_α4 receptors, the EC50 for cytosine or TC-2559 was not different from wild type, but the EC50 for varenicline was 4-fold lower (Table 1). In addition, the competitive antagonist dihydro-b-erythroidine (DhßE) inhibited the responses activated by ACh with wild type potency (IC50 = 0.25 (0.07-0.43) μM versus wild type IC50 = 0.28 (0.024-0.033) μM; mean (minimum;maximum for 95% CI)). By comparison, β2L146C_α4_β2_α4_α4 receptors retained wild type sensitivity for varenicline but displayed reduced sensitivity for cytosine and TC-2559 (Table 1). These results indicate that α4T152C and β2L146C on loop E of the complementary component of the agonist site at the α4/α4 and α4/β2 interfaces, respectively, affect agonist sensitivity. The findings are in agreement with a previous report that noted that L119C, L119C, and L121C, the corresponding cysteine substitutions in the vertebrate muscle nAChR subunits γ, ε, and δ, respectively, treated with an MTS reagent, display reduced affinity for dimethyl-d-tubocurarine and α-conotoxin M1 (27). The relative efficacies of agonists, compared with ACh, were not affected by the α4T152C or β2L146C substitutions (Table 1). Overall, the findings indicate that cysteine substitution of α4T152 in the agonist site at the α4/α4 interface or β2L146 in the agonist site at the α4/β2-1 interface is tolerated.

**Rates of MTSET Reaction with Substituted Cysteines**—We tested first whether the substituted cysteines were accessible to MTSET using the protocol depicted in Fig. 2A. Exposure to a saturating concentration (1 mM) of MTSET decreased the ACh...
(EC50 × 5, 1 mM) responses of β2_α4/β2_α4/T152C_α4 receptors by 43 (56;30)% (mean (95% CI)) (Fig. 2, B and D, and Table 2). In contrast, MTSET decreased the ACh (EC50 1.6 mM) responses of β2_1446C_α4/β2_α4/α4 receptors by 71 (87;56)% (mean (95% CI)) (Fig. 2, C and D, and Table 2). Longer periods of MTSET applications produced no further changes in the ACh responses. Differences in the levels of ACh response reductions induced by MTSET suggest that both types of agonist sites contribute differentially to receptor activation. The ACh responses of wild-type receptors were not affected by exposure to MTSET (Fig. 2D and Table 2), demonstrating that the changes in the amplitude of agonist responses of the mutant receptors were due to MTSET reaction with the substituted cysteines. Exposure to 1 mM MTSET also decreased the responses of β2_α4/β2_α4/T152C_α4 to cytisine (EC50 × 5 = 70 μM) or varenicline (EC50 × 5 = 12 μM) (Fig. 2, E and F), and the extent of the decrease was comparable with that observed for the responses of ACh in this mutant receptor (Table 2). Exposure to the reducing agent dithiothreitol (DTT) (1 mM; 120 s) fully reversed the effects of MTSET on the ACh responses of β2_α4/β2_α4/T152C_α4 receptors. This shows that any modification of endogenous disulfide bonds in β2_α4/β2_α4/T152C_α4 receptors by DTT had minimal effects on receptor function. In contrast, DTT (1 mM, 120 s) had no effects on the responses of β2_1446C_α4/β2_α4/α4 receptors (Fig. 2, B and C), further indicating differences between the agonist sites. Longer DTT applications had lethal effects on oocytes.

Next, we determined the rates of covalent modification of the introduced cysteines by measuring the effect of successive saturating applications of MTSET on ACh current responses using the protocol described under “Experimental Procedures.” The decrease in ACh current responses was plotted against cumulative duration of MTSET exposure and fit with a single exponential decay curve, which yields a pseudo-first-order rate constant (k1). To correct for the concentration dependence of the rate (21), a second order rate constant (k2) (Table 3) was calculated by dividing k1 by the concentration of MTSET used. This correction was needed to compare the rate of MTSET reactions on both types of agonist sites. For both mutant receptors and for all agonists tested, except cytisine on β2_1446C_α4/β2_α4/α4 receptors, the maximal effects of MTSET observed in the rate experiments were consistent with those measured using maximal concentrations of MTSET, indicating that the MTSET reactions went to completion. When cytisine was used to stabilize the ACh responses of β2_1446C_α4/β2_α4/α4 receptors, the maximal effects of MTSET were ∼2.3-fold smaller than the maximal effect observed when any of the other agonists were used to stabilize the ACh current responses of β2_1446C_α4/β2_α4/α4 receptors (Fig. 3F). Application of 1 mM MTSET at the end of the cytisine protection assay did not increase the plateau (not shown). These findings suggest that prolonged exposure to cytisine produces long-term desensitization in a subset of β2_1446C_α4/β2_α4/α4 receptors, which could make these receptors unavailable for MTSET reaction. This possibility is consistent with previous studies, which have shown that repeated exposure of α4/β2 nAChRs to submaximal concentrations of cytisine elicits high-affinity, long-term desensitization of receptor function (28). For both mutant receptors, the control MTSET reaction rate was not affected by the application of any of the agonists tested, including cytisine on β2_1446C_α4/β2_α4/α4 receptors, during the stabilization phase of the rate protocol used (Fig. 3A and Table 3), which proves that any changes in the responses to ACh pulses observed during the protection assays are due to changes in MTSET reaction rates. Comparison of the calculated k2 values indicated that the fastest reaction of MTSET occurred at α4T152C (Table 3), suggesting that this residue is more accessible than β2L146C to covalent modification by MTSET.

Agonist Sites Have Different Ligand Selectivity—To determine the ligand selectivity of the agonist site at the α4/α4 and α4/β2-1 interfaces, established (α4/β2)_α4 agonists (i.e. ACh, cytisine, varenicline, TC-2559, and sazetidine-A) (29) were tested for the ability to protect the substituted cysteines from MTSET reactions. We reasoned that co-application with agonists should decrease the rate of MTSET reaction with substituted cysteines if the co-applied agonists competed with this reagent for access to the cysteine-substituted agonist sites. In
Agonist Efficacy and Non-equivalence of Agonist Sites

**TABLE 2**

| Effect | Percentage Change of ACh Response to MTSET | P Value |
|--------|-------------------------------------------|---------|
| ACh    | -13.7% (Δ%; 8+5), n = 8                   | 0.01    |
| ACh    | -71 (Δ%; -56+5), n = 5                    | 0.001   |
| ACh    | -43.00 (Δ%; -56+30), n = 5                | 0.001   |
| Var    | -51 (Δ%; -32+6), n = 6                    | 0.05    |
| Var    | -51 (Δ%; -40+7), n = 7                    | 0.05    |
| ACh    | -48 (Δ%; -55+41), n = 12                  | 0.05    |
| TC-2559| -74 (Δ%; -32+55), n = 4                   | 0.05    |
| Saz-A  | -88 (Δ%; -80+9), n = 4                    | 0.05    |

Agreement with our previous studies that showed binding of ACh to the α4/β2 and α4/α4 interfaces (11), ACh protected α4T152C and β2L146C from MTSET reactions (Fig. 3, C and D). Cytisine and varenicline also protected the agonist sites at the α4/β2 and α4/α4 interfaces (Fig. 3, E–H). Protection could have also occurred if occupation of the agonist sites by ACh, cytisine, or varenicline induced structural rearrangements, resulting in a decreased accessibility of the substituted cysteines to MTSET. To test this possibility, we determined the effect of DhβE, a competitive antagonist that is thought not to cause agonist-like conformational changes in the agonist binding site of α4β2 nAChRs (30) and which we have shown previously to bind the agonist sites at the α4/β2 and α4/α4 interfaces (11). In agreement with our previous studies (11), DhβE was effective at protecting α4T152C from covalent modification by MTSET.
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| Protec tant | Control rate $k_3$ | Protection assay $k_{3C/k3P}$ |
|-------------|-------------------|-------------------------------|
| **β2**4/4β2,1512C | | |
| ACh | 100 ± 15 | 6 | 21 ± 7.5 | 6** | 4.8 |
| Cyt | 103 ± 16 | 5 | 6 ± 0.8 | 3** | 16 |
| Var | 158 ± 58 | 4 | 27 ± 7.8 | 4* | 5.9 |
| TC-2559 | 103 ± 36 | 3 | 123 ± 57 | 5 | 0.84 |
| Saz-A | 133 ± 39 | 4 | 146 ± 42 | 6 | 0.91 |
| DkME | 87 ± 15 | 4 | 3.7 ± 0.5 | 3* | 23.5 |
| **β2**4/4β2,1512C,1424A | | |
| ACh | 6.4 ± 0.9 | 4 | 1.4 ± 0.5 | 5* | 4.6 |
| Cyt | 9.2 ± 8.7 | 7 | 0.76 ± 0.15 | 3* | 12 |
| Var | 5 ± 1 | 5 | 0.75 ± 0.35 | 3* | 6.7 |
| TC-2559 | 3.5 ± 0.5 | 4 | 1 ± 0.4 | 4* | 3.5 |
| Saz-A | 4.0 ± 0.6 | 4 | 1.5 ± 0.48 | 4* | 2.7 |

(A) For all three mutant receptors shown, statistical differences were determined by Student’s t tests or one-way analysis of variance. **p < 0.01; *** p < 0.001. ° Statistical differences between the control rate constants determined under control and protected assay conditions (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

In contrast, TC-2559 and sazetidine-A protected only **β2**4/4β2,1512C,1424A. Accordingly, alanine substitution of αH142 in the complementary face of the agonist site at the α4/4 interface would allow TC-2559 and sazetidine-A access to the α4/4 interface, which should in turn increase the efficacy of activation of (**β2**4/4α)4 receptors by these agonists. Fig. 5 (C-H) shows that the efficacy of TC-2559 and sazetidine-A relative to that of ACh at **β2**4/4α4,1424A α4 receptors increased by 4- and 5-fold, respectively (Table 1). To exclude the possibility that the increased efficacy of TC-2559 and sazetidine-A may reflect non-local effects (e.g. allosteric effects), we engineered mutant **β2**4/4α4,1512C,1424A α4 receptor and tested if these compounds could slow down the reaction of MTSET with **α4**1512C. Fig. 6A shows that **β2**4/4α4,1512C,1424A α4 receptors were sensitive to ACh, TC-2559, and sazetidine-A. The concentration response effects of ACh, TC-2559, and sazetidine-A at **β2**4/4α4,1512C,1424A α4 receptors were comparable with that observed at **β2**4/4α4,1512C,1424A α4, indicating that incorporation of T152C into the complementary face of the agonist site at the α4H142A/α4 interface is well tolerated (Fig. 6, A and B, and Table 1). Exposure to 1 mM MTSET decreased the amplitude of the peak responses to ACh (1.15 mM), TC-2559 (20 μM), and sazetidine-A (20 μM) by 48 (55:41) (mean (95% CI)), 74 (32:55%) (mean (95% CI)), and 88 (80:91%) (mean (95% CI)), respectively (Fig. 6C). In the case of TC-2559 and sazetidine-A, 1 mM MTSET reduced the peak current responses to amplitudes typical of those evoked by maximal concentrations of these ligands at wild type **β2**4/4α4,1512C,1424A α4 receptors (Table 1). Furthermore, as shown in Fig. 6, D and E, the rate of MTSET reaction with **β2**4/4α4,1512C,1424A α4 receptors was significantly slowed in the presence of TC-2559 or sazetidine-A (Table 3 for the estimated rate values). These findings indicate that TC-2559 and sazetidine-A bind the agonist site at the α4/4 interface when the H142A mutation is present in the complementary component of this agonist site.

**DISCUSSION**

We (11) and others (12) have shown previously that the (**β2**4/4α)4 nAChR contains three functional agonist binding sites for ACh. Binding of ACh to two sites produces effective gating, but engagement of the third agonist site produces maximal activation. Two of the sites are located at α4/β2 interfaces and are thus structurally identical. The third agonist site is at the α4/α4 interface, where it occupies a position homologous to that of the agonist sites at α4/β2 interfaces. We wanted to investigate whether the agonist sites at α4/α4 and α4/β2 interfaces have differing ligand selectivity and whether such differences could impact agonist efficacy at (**β2**4/4α)4 nAChRs.

Our findings indicate that the agonist site at the α4/α4 interface is a
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A key determinant of agonist efficacy; occupancy of this site increases agonist efficacy, whereas exclusion from the site leads to partial agonism. This is likely to apply to other pentameric ligand gated ion channels assembled from α and β subunits into a (αβ)2α receptor stoichiometry (e.g. (α3β4)2,α3 (18) and heteromeric (αβ)2α glycine receptors (19)). Given that the two stoichiometries of the α4β2 nAChR may express in neurons (31–35), the findings may encourage a fresh outlook and new impetus in the design of α4β2-targeted compounds.

From the observations of 1) the effects of the cysteine substitutions on the sensitivity of the receptors to agonists, 2) the effects of saturating concentrations of MTSET on the amplitude of the current responses to ACh, 3) the extent of DTT-driven recovery from MTSET reactions, 4) the effects of cysteine on the maximal effects of MTSET, and 5) the rates of MTSET reaction and the extent of agonist-dependent protection from MTSET reactions, we confirm that the sites at α4/α4 and α4/β2 interfaces in (α4β2)2α4 nAChRs are functionally non-equivalent (11, 17). Functional non-equivalence of the agonist sites may be attributed largely to differences in the complementary site of the agonist sites. Because the principal face is contributed by α4 subunits at both types of binding sites, binding interactions between agonists and the principal face of the agonist sites are possibly similar in the two sites. In contrast, the complementary face is contributed by the α4 subunit in the α4/α4 interface and by a β2 subunit in the α4/β2 interface, which probably provides interface-specific structural elements for agonist site-ligand interactions.

A key finding of our studies is that the agonist site at the α4/α4 interface, but not the agonist sites at α4/β2 interfaces, excludes agonists of a certain size. Comparisons of the MCL and ASA of agonists suggest that the site at the α4/α4 interface is readily occupied by small ligands (e.g. ACh, cytisine, and varenicline). Larger agonists (e.g. TC-2559 or sazetidine-A) cannot enter the site, constraining these compounds to trigger ion channel gating through the agonist sites at α4/β2 interfaces only. A single residue on the complementary face of the site at the α4/α4 interface, α4H142, impedes entry to agonists with a critical size, thus acting as a molecular sieve in the agonist site at the α4/α4 interface and, hence, as a key determinant of partial agonism at (α4β2)2α4 nAChR. Given that maximal activation of (α4β2)2α4 nAChRs increases by additional binding to the agonist site at the α4/α4 interface (11, 12), exclusion from the process of desensitization that may have occurred during the assay. B, current traces showing the protection assay protocol. The stabilization phase of the protection assay was as in A, but during the protection phase, the protectants were co-applied with MTSET. C–I, normalized ACh currents in the absence and presence of agonist on β2_α4β2_α4T152C_α4 (left) or β2_1464C_α4_β2_α4_α4 (right) receptors. ACh (C and D), Cyt (E and F), Var (G and H), Saz-A (J, L, and J), and TC-2559 (K and L) were plotted versus cumulative time of MTSET and fit with single exponential functions (see "Experimental Procedures"). Data points were normalized to ACh currents at time 0 and are the mean ± S.E. of at least four experiments. For β2_α4_β2_α4T152C_α4, the concentrations of agonists used were as follows: EC50 × 5, ACh, 1 μM; Cyt, 55 μM; Var, 12 μM; TC-2559, 15 μM. For β2_α4_β2_α4T152C_α4 receptors, the concentrations of Var (45 μM) and TC-2559 (65 μM) were also EC50 × 5, but for Cyt and ACh, the concentrations were 0.3 μM (EC100) and 1.6 μM (EC50), respectively. For both mutant receptors, the concentration of Saz-A used was 1 μM, a concentration that produced maximal effects (EC100) in wild type receptors (13). Error bars, S.E.
FIGURE 4. Docking of nicotinic ligands to α4/α4 and α4/β2 interfaces in a (α4β2)_α4 receptor homology model. Single docking of ACh (A and B), Cyt (C and D), and Var (E and F) at the α4/α4 and α4/β2 interfaces. G and H show the docking Saz-A and TC-2559 to the homology model of the α4/β2 interface. Agonists docked in the homology models of the α4/β2 interface are shown as sticks. I, MCL and ASA of agonists of (α4β2)_α4 nACHRs. ASA (Å²) values were calculated were calculated using ChemAxon software, whereas the MCL (Å) of the agonists was measured after energy minimization using spartan’08 software.

FIGURE 5. Comparison of homology models of α4/β2 and α4/α4 interfaces. Superimposition of structural homology models of non-occupied α4/α4 interface with α4/β2 interface engaged by Saz-A (A) or TC-2559 (B). In each view, β2V136 and α4H142 are shown as sticks. C and D, representative traces of the effects of Saz-A and TC-2559 on wild type or β2_α4_β2_α4_α4 receptors. The concentration-response curves for these agonists at wild and mutant receptors (E–H) were generated from at least eight experiments. Error bars: S.E.

agonist site at the α4/α4 interface inexorably leads to partial agonism.

Binding to all three sites, however, does not necessarily lead to maximal activation. Thus, neither cytisine nor varenicline...
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behave as full agonists at (α4β2)_2α4 nAChRs, although they are capable of occupying all agonist sites in this receptor stoichiometry. Considering that the α4/β2 and α4/α4 interfaces have different complementary components, interactions with the complementary interface probably define the agonist efficacy of agonist able to occupy all agonist sites in (α4β2)_2α4 nAChRs. In accord with this possibility, we found that the extent of protection of the substituted cysteines given by agonists, which partly depends on the affinity of agonists for the cysteine-substituted sites, was subunit interface-dependent. These findings add further support to previous suggestions that residues contributed by the complementary subunit critically influence agonist efficacy (3–10).

An unexpected observation was that the efficacy of TC-2559 and sazetidine-A at (α4β2)_2α4 nAChRs is not (α4β2)_2-like, although these compounds bind only the agonist sites at α4/β2 interfaces in both receptor types, the only type of operational agonist sites present in (α4β2)_2β2 receptors (11). At (α4β2)_2β2 nAChRs, TC-2559 behaves as a superagonist (13, 14), whereas sazetidine-A acts as a full agonist (13). A likely explanation is that agonist sites at α4/β2 interfaces in (α4β2)_2β2 and (α4β2)_2α4 nAChRs are functionally distinct despite being structurally identical. In support of this possibility, it has been reported recently that cytisine, an agonist that displays strong functional differences on the alternate α4β2 nAChRs (13, 14), shows receptor stoichiometry differences for hydrogen-bonding interactions with the β2 subunit (9).

How could structurally identical agonist sites be functionally non-equivalent? Functional non-equivalence could arise by different subunit environments surrounding the agonist binding sites, as suggested for the α1β2γ2, GABA_A receptor (36). In the case of the alternate α4β2 nAChRs, it may be that functionally relevant interactions between the fifth subunit and neighboring subunits are receptor-specific, which could influence agonist effects. For example, in addition to contributing the primary side of an operational agonist site, the fifth subunit in the (α4β2)_2α4 nAChR could influence binding of agonists to the sites at α4/β2 interfaces such that agonist binding poses to those sites are different from those at the (α4β2)_2β2 stoichiometry. Some of these interactions could be weakened or strengthened upon agonist binding, and this could influence the ability of occupied receptors to reach the shut states immediately preceding gating (flipped or priming states) (37, 39). It has been suggested that full and partial agonists differ in that partial agonists are less effective than full agonists in inducing flipped receptor states (37). The suggestion that the fifth subunit may be directly involved in the coupling of agonist binding to ion channel gating is consistent with recent electron microscopy studies of Torpedo marmorata nAChRs that suggest that displacement of the accessory subunit (β1), driven by motions of the inner and outer sheets of the β barrel of neighboring agonist binding α1 (α1_) induced by agonist binding, plays a greater determining role in gating than hitherto thought (39). We show here that in the case of the (α4β2)_2α4 nAChR, the fifth subunit critically shapes the functional properties of this receptor type.

We are unable at this time to propose an adequate kinetic model for the activation/desensitization cycle of the (α4β2)_2α4 nAChR by agonists, because single channel data for this receptor are lacking. Nevertheless, on the basis that the (α4β2)_2α4 receptor efficiently activates when only two agonist sites are engaged by ACh and that these partially occupied receptors activate and desensitize with affinities comparable with those determined for the (α4β2)_2β2 receptor (17), we propose that the (α4β2)_2α4 can display (α4β2)_2-like or (α4β2)_2α4 activity, depending on the concentration of ACh. This implies that there are two pathways for the activation of (α4β2)_2α4 nAChRs (Fig. 7, A and B). For simplicity, we have omitted from the scheme shown in Fig. 7, A and B, open and desensitized recep-

FIGURE 6. Sazetidine-A and TC-2559 effects on β2_α4 β2_α4

A and B, concentration-response curve for TC-2559 or Saz-A stimulated current responses at β2_α4 β2_α4 receptors. Each data point represents the mean ± S.E. (error bars) for four independent studies. The estimated values for EC50 and efficacy are shown in Table 1. C, histogram showing the effects of 1 mM MTSET on the current responses of β2_α4 β2_α4 receptors to TC-2559 and Saz-A (20 μM). The percentage change in the responses to ACh, TC-2559, and Saz-A is defined as (I(agonist + MTSET)/I(agonist) - 1) × 100. Data represent the mean of 12 (ACh) or 3 (TC-2559 and Saz-A) independent experiments. See Table 2 for statistical analysis of the data. D and E, rates of reaction of MTSET at β2_α4 β2_α4 receptors to EC50 × 5 applications of ACh (1.15 mM), TC-2559 (20 μM), and Saz-A (20 μM). The percentage change in the responses to ACh, TC-2559, and Saz-A is defined as (I(agonist + MTSET)/I(agonist) - 1) × 100. Data represent the mean ± S.E. of at least four experiments. The concentration of TC-2559 and Saz-A used during the stabilization of the responses to ACh for correction for any process of desensitization that may have occurred during the assay. Normalized ACh currents in the absence and presence of TC-2559 (‡) or sazetidine-A (§) were plotted versus cumulative time of MTSET and fit with single exponential functions (see “Experimental Procedures”). Data points were normalized to ACh currents at time = 0 and are the mean ± S.E. of at least four experiments. The concentration of TC-2559 and Saz-A used during both the stabilization of the EC50 × 5 ACh (1.15 mM) responses and the protection assays was 20 μM.
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