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An Accessory Agonist Binding Site Promotes Activation of α4β2* Nicotinic Acetylcholine Receptors*

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Background: In $(\alpha4\beta2)_2\alpha4$ nicotinic acetylcholine receptors, there is an agonist binding site at the $\alpha4/\alpha4$ subunit interface.
Results: $\alpha2$, $\alpha3$, and $\alpha6$ accessory subunits can form an agonist site with $\alpha4$. These promote activation upon agonist binding at $\alpha4/\beta2$ agonist sites.
Conclusion: Accessory subunit agonist sites greatly influence receptor function.
Significance: These sites are promising drug targets.

Neuronal nicotinic acetylcholine receptors containing α4, β2, and sometimes other subunits (α4β2* nAChRs) regulate addictive and other behavioral effects of nicotine. These nAChRs exist in several stoichiometries, typically with two high affinity acetylcholine (ACh) binding sites at the interface of α4 and β2 subunits and a fifth accessory subunit. A third low affinity ACh binding site is formed when this accessory subunit is α4 but not if it is β2. Agonists selective for the accessory ACh site, such as 3-[3-(3-pyridyl)-1,2,4-oxadiazol-5-yl]benzonitrile (NS9283), cannot alone activate a nAChR but can facilitate more efficient activation in combination with agonists at the canonical α4β2 sites. We therefore suggest categorizing agonists according to their site selectivity. NS9283 binds to the accessory ACh binding site; thus it is termed an accessory site-selective agonist. We expressed $(\alpha4\beta2)_2$ concatamers in *Xenopus* oocytes with free accessory subunits to obtain defined nAChR stoichiometries and α4/accessory subunit interfaces. We show that α2, α3, α4, and α6 accessory subunits can form binding sites for ACh and NS9283 at interfaces with α4 subunits, but β2 and β4 accessory subunits cannot. To permit selective blockage of the accessory site, α4 threonine 126 located on the minus side of α4 that contributes to the accessory site, but not the α4β2 sites, was mutated to cysteine. Alkylation of this cysteine with a thioreactive reagent blocked activity of ACh and NS9283 at the accessory site. Accessory agonist binding sites are promising drug targets.

nAChRs[2] contain five homologous subunits organized to form a central cation channel whose opening is gated by the binding of ACh. Homomeric $\alpha7$ nAChRs have five $\alpha7$ subunits and five ACh binding sites at their extracellular interfaces (1). Heteromeric $\alpha4\beta2$ nAChRs assemble into $(\alpha4\beta2)_2\alpha4$ or $(\alpha4\beta2)_2\beta2$ stoichiometries with three or two ACh binding sites (2, 3). Binding of ACh to one site is able to activate, inefficiently, both homomeric and heteromeric nAChRs (4, 5). Binding to two sites in an $\alpha7$ nAChR is more efficient, and binding to three is most efficient for activation. By contrast, binding to four or five sites in $\alpha7$ nAChRs promotes desensitization more rapidly than activation (6). In $\alpha4\beta2$ nAChRs, ACh can bind at the interface between $\alpha4$ and $\beta2$ subunits (abbreviated as $\alpha4/\beta2$) where the $\alpha4$ subunit on the left forms the plus face of an agonist site and the $\beta2$ subunit on the right forms the minus face. In the $(\alpha4\beta2)_2\alpha4$ stoichiometry, a third low affinity ACh site is present at the $\alpha4/\alpha4$ interface (2, 3). This results in 4-fold larger responses evoked by ACh of $(\alpha4\beta2)_2\alpha4$ stoichiometry than the $(\alpha4\beta2)_2\beta2$ stoichiometry. Because the high affinity $\alpha4/\beta2$ agonist sites contribute less than 25% of the total response of $(\alpha4\beta2)_2\alpha4$ nAChRs, these nAChRs appear to be low affinity if the high affinity component is not clearly resolved (7, 8). Actually, $(\alpha4\beta2)_2\alpha4$ nAChRs can be activated by low concentrations of agonists from their intrinsic $\alpha4/\beta2$ sites to the same extent as the $(\alpha4\beta2)_2\beta2$ nAChRs (2).

NS9283 is representative of a new class of selective agents targeting $\alpha4\beta2$ nAChRs that have proven useful in aiding nAChR agonists in reducing neuropathic pain and improving cognition (9–15). It has been termed a positive allosteric modulator (PAM) because it cannot activate nAChRs by itself but enhances $\alpha4\beta2$ nAChR activity in response to agonist stimulation (9, 10, 12). However, it was recently established that NS9283 is neither allosteric nor a modulator. It is not allosteric because NS9283 acts as a selective agonist at the ACh binding site formed at the $\alpha4/\alpha4$ interface (16, 17). NS9283 cannot activate through its action on the $\alpha4/\alpha4$ site alone. In combination with agonists at $\alpha4/\beta2$ sites, it produces the high probability of channel opening resulting from increased binding site occupancy (12, 17). Because NS9283 achieves its effect by occupying a third ACh site, just as any other full agonist would at that site, it is not a modulator. NS9283 does not exceed the maximum

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[2] The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; DMSO, dimethyl sulfoxide; MTSEA, (2-aminoethyl)methanethiosulfonate; NS9283, 3-[3-(3-pyridyl)-1,2,4-oxadiazol-5-yl]benzonitrile; PAM, positive allosteric modulator; SSAg, site-selective agonist; EC, effective concentration.

activation efficiency of $(\alpha4\beta2)_2\alpha4$ nAChRs by ACh (18). As expected, NS9283 is without effect on $(\alpha4\beta2)_2\beta2$ nAChRs for lack of the $\alpha4/\alpha4$ binding site (17, 19).

In addition to NS9283, other well known $\alpha4\beta2^*$ nAChR ligands also show differential actions on $\alpha4/\beta2$ and $\alpha4/\alpha4$ agonist sites. The drug sazetidine is a full agonist at $\alpha4/\beta2$ primary agonist sites but does not bind to the $\alpha4/\alpha4$ accessory ACh binding site (20–22). Cytisine acts at both types of ACh binding sites as a partial agonist (16, 20, 22). Dihydro-β-erythroidine is an antagonist at both $\alpha4/\beta2$ and $\alpha4/\alpha4$ ACh binding sites (3). These orthosteric ligands are distinguished from PAMs that act at non-orthosteric ACh binding sites, such as the C terminus of $\alpha4$ (23), or transmembrane sites on $\alpha7$ nAChRs (24–26).

To avoid misleading nomenclature and provide nomenclature that reflects the mechanism of action, we suggest designating sazetidine and NS9283 as ACh binding site site-selective agonists (SSAgs). There may also be site-selective antagonists. Based on specific site selectivity, SSAgs can be further divided into two groups: accessory SSAgs, such as NS9283, and primary SSAgs, such as sazetidine. Here we confirm and extend what is known about accessory site activation of $\alpha4\beta2^*$ nAChRs (which contain $\alpha4$, $\beta2$, and possibly other subunits).

Because $\alpha4$ and $\beta2$ subunits can form conventional ACh binding sites, they are referred to as structural subunits. The fifth subunit of $\alpha4\beta2^*$ nAChRs, which may or may not form an ACh site depending on the subunit, is referred to as an accessory subunit. Some subunits like $\alpha4$ and $\beta2$ can function as both structural and accessory subunits, whereas others like $\alpha5$ and $\beta3$ usually function only as accessory subunits. Here we investigate the ability of $(\alpha4\beta2)_2^*$ nAChRs to form ACh and NS9283 binding sites with the accessory subunits $\alpha2$, $\alpha3$, $\alpha4$, $\alpha6$, $\beta2$, and $\beta4$. Functional impairment by blocking accessory sites suggests that accessory agonist sites exist and that they promote channel activation from the interface between several α subunits and $\alpha4$.

Experimental Procedures

Chemicals—2-Aminoethyl methanethiosulfonate (MTSEA) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). NS9283 was synthesized as described previously (27). A 10 mM stock of NS9283 was prepared in dimethyl sulfoxide. Dilutions of NS9283 and MTSEA were prepared daily in testing buffer before use. All other chemicals were purchased from Sigma-Aldrich unless otherwise noted.

cDNAs and cRNAs—Human $\alpha3$, $\alpha4$, $\alpha6$, $\beta2$, and $\beta4$ were cloned in this laboratory (28–31). The human $\alpha2$ subunit was obtained from OriGene Technologies, Inc. (Rockville, MD). The $\alpha2$ sequence was cut out with the restriction enzymes SmaI and XhoI to shorten the untranslated region and improve functional expression. The 2.0-kb DNA fragment coding for $\alpha2$ was subcloned into the pSP64 vector for RNA preparation or into pcDNA3.1/Zeo(+) (Invitrogen) for human cell transfection.

Syntheses of concatamers of $\beta2(AGS)_6\alpha4$ (abbreviated as $\beta2$-$\alpha4$) and $\beta2(AGS)_6\alpha4(AGS)_{12}\beta2(AGS)_6\alpha4$ (abbreviated as $\beta2$-$\alpha4$-$\beta2$-$\alpha4$) were described previously (32, 33). Signal peptides of $\alpha4$, $\alpha6$, and $\beta2$ subunits were analyzed by Signal-3L (34). The mature amino acid sequences were used to number $\alpha4$, $\alpha6$, and $\beta2$ subunits. Mutations in the dimeric concatamer are numbered as they are in single subunits and displayed in the upper right corner of the subunit that carries the mutation. For example, $\beta2$-$\alpha4^{T126C}$ means that the threonine at the 126 position of the $\alpha4$ subunit is replaced by a cysteine. Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The amino acids mutated in $\alpha4^{T126C}$, $\beta2^{L121C}$, and $\alpha6^{L250S}$ are underlined: $\alpha4$, VQW<u>T</u>PPAI; $\beta2$, IFW<u>L</u>PPAI; $\alpha6$, SVL<u>L</u>SLTV. All mutations were confirmed by sequencing.

After linearization and purification of cDNAs, RNA transcripts were prepared *in vitro* using mMessage mMachine kits (Ambion, Austin, TX). Concentrations of cDNAs and cRNAs were calculated by spectrophotometry.

Oocyte Removal and Injection—Oocytes were removed surgically from *Xenopus laevis* as described except that a higher concentration (0.26 mg/ml) of collagenase type IA (Sigma) was used to obtain oocytes optimal for the automated voltage clamp instrument described later.

Oocyte injections were performed within 48 h after surgery. Oocytes were injected with 20 ng of $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ concatamer cRNA and free single subunit at a 1:1 ratio except with $\alpha6$ and $\beta2$ subunits. To express $(\alpha4\beta2)_2\alpha6$ and $(\alpha4\beta2)_2\beta2$ in oocytes with high enough currents, 40 ng of total cRNA was injected in each oocyte at a 1:1 ratio of concatamer $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ to $\alpha6$ subunit, and 30 ng of total cRNA was injected at a 2:1 ratio of concatamer $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ to $\beta2$ subunit. Dimeric concatamer $\beta2$-$\alpha4$ produced larger currents than tetrameric concatamers when expressed with free subunits. To obtain functional responses above 0.1 μA but below 20 μA, 1.25 ng of dimeric concatamer was co-injected with 1.25 ng of free subunit ($\alpha2$, $\alpha3$, $\alpha4$, and $\beta4$) per oocyte. 10 ng of total cRNAs were injected for the $\alpha6$ or $\beta2$ subunit when co-expressed with $\beta2$-$\alpha4$. Function was assayed 3–7 days after injection.

Electrophysiology—Currents in oocytes were measured using a manual two-electrode voltage clamp amplifier setup (oocyte clamp OC-725, Warner Instrument, Hamden, CT) or OpusXpress 6000A (Molecular Devices, Sunnyvale, CA) (30, 35). OpusXpress is an integrated system that provides automated impalement, voltage clamp, and drug delivery for up to eight oocytes in parallel (35). Electrodes were filled with 3 M KCl and had resistances of 0.5–10 megaohms for the voltage electrode and 0.5–3 megaohms for the current electrode. Oocytes were voltage-clamped at a holding potential of −50 mV. Data were collected and filtered at 50 Hz. 200 μl of drugs were delivered on top of oocytes for 4 s through the sidewall of the bath to minimize disturbance. Between drug applications, oocytes received a 30-s prewash and 223-s postwash with ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) plus 0.5 μM atropine perfused through the bath at a rate of 3 ml/min unless otherwise noted.

In concentration/response experiments, each oocyte received two initial control applications of ACh (300 μM for concatamer expressed with free α subunits and 30 μM for concatamer expressed with free β subunits) followed by application of various concentrations of ACh (from low to high). In MTSEA experiments, each oocyte received 2 mM MTSEA for 60 s at the rate of 0.9 ml/min and then was incubated with MTSEA for an additional 5 min while the ND96 wash was stopped to retain the

reagent in the bath before being washed with buffer solution for 287 s. Oocytes were discarded after experiments involving MTSEA because they had been covalently modified. In NS9283 experiments, 10 μM NS9283 was preapplied to oocytes expressing $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ and free subunits for 30 s at the rate of 1.8 ml/min and incubated for an additional 80 s in a static bath before its co-application with ACh for 4 s at 3 ml/min at EC_{20-40} concentrations (30 μM for α subunits, 1 μM for $\beta2$ subunit, and 3 μM for $\beta4$ subunit). Preincubation with NS9283 eliminates kinetic effects of NS9283 binding, leaving only the kinetics of ACh responses in nAChRs at equilibrium with NS9283. Control experiments were performed on the same oocytes before NS9283 applications following the same protocol in which 10 μM NS9283 was replaced with 0.1% (v/v) DMSO. Potentiation by NS9283 was calculated by increased response to ACh with 10 μM NS9283 *versus* response of ACh co-applied with 0.1% DMSO. In the ACh concentration/response curve experiment with NS9283, 10 μM NS9283 was co-applied with different concentrations of ACh without preapplication to shorten the experiment duration.

The peak amplitudes of experimental responses were calculated relative to the maximum ACh response or the average of the first two control ACh responses to normalize the data and compensate for variable expression levels among oocytes. Mean and S.E. were calculated from normalized responses. The numbers of oocytes tested are listed. The Hill equation was fit to the concentration/response relationship using a nonlinear least squares curve fit method (Kaleidagraph, Abelbeck/Synergy, Reading, PA): $I(x) = I_{max}\,[x^{nH}/(x^{nH} + EC_{50}{}^{nH})]$ where $I(x)$ is the peak current measured at the agonist concentration x, I_{max} is the maximum current peak at the saturating concentration, EC_{50} is the agonist concentration required to achieve half of the maximum response, and n_H is the Hill coefficient.

Cell Culture and Transfection—All cells were maintained as described previously (29). The human embryonic kidney tsA201 (HEK) cell lines stably expressing human $\alpha4\beta2$ and $\alpha3\beta2$ were described previously (8, 29, 36). To establish stable cell lines of $\alpha3\beta4$, $\alpha2\beta2$, $\alpha2\beta4$, and $\alpha4\beta4$, equal amounts of plasmid encoding appropriate α and β subunits were transfected into HEK cells using the FuGENE 6 transfection reagent (Roche Diagnostics) at a ratio of 6 μg of DNA/18 μl of FuGENE 6/100-mm dish. A single colony expressing appropriate nAChRs was selected as described previously (36).

FLEXstation Experiments—For functional tests of nAChRs expressed in HEK cells, we used a FLEXstation (Molecular Devices) bench top scanning fluorometer as described by Kuryatov *et al.* (36). To increase the expression level of $\alpha2\beta2$ and $\alpha3\beta2$ nAChRs, the plates were incubated at 29 °C for 20 h before being tested. The membrane potential kit (Molecular Devices) was used according to the manufacturer's protocols. Serial dilutions of NS9283 were manually added to the assay plate 15 min prior to recording. ACh dilutions were prepared in V-shaped 96-well plates (Fisher Scientific Co.) and added in cell culture wells at 20 μl/s during recording. Each data point was averaged from three to four responses from separate wells. The potency and efficacy of drugs were calculated from the Hill equation described above.

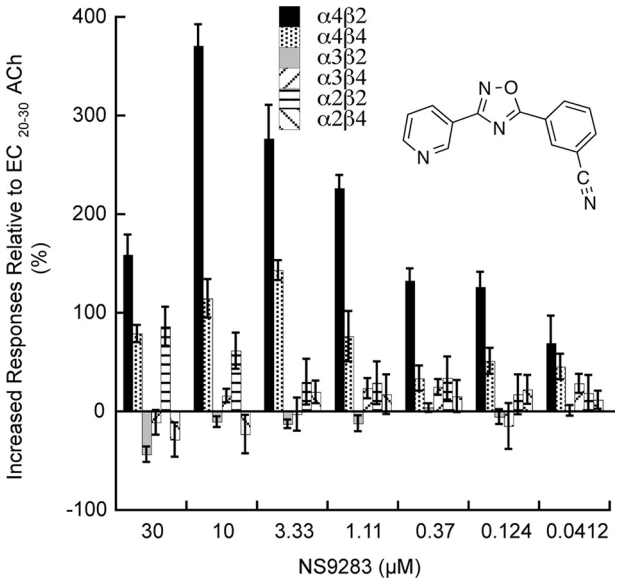

FIGURE 1. **Chemical structure and potentiation effect of NS9283 on activation of nAChR subtypes expressed in HEK cells.** Various concentrations of NS9283 were preapplied to cell lines for 15 min before acute application of ACh at EC_{20-30} concentration (*i.e.* $\alpha4\beta2$, 0.4 μM; $\alpha4\beta4$, 1 μM; $\alpha3\beta2$, 4 μM; $\alpha3\beta4$, 5 μM; $\alpha2\beta2$, 0.4 μM; $\alpha2\beta4$, 0.8 μM). A fluorescent indicator was used to record the membrane potential changes. NS9283 potentiates $\alpha4\beta2$, $\alpha4\beta4$, and $\alpha2\beta2$ nAChRs but not $\alpha2\beta4$, $\alpha3\beta2$, or $\alpha3\beta4$ nAChR. Because NS9283 inhibited its own potentiation at higher concentrations, we could not fit the concentration/response curve into the Hill equation to obtain the exact potency or maximum efficacy values of NS9283 for all nAChR subtypes tested. Therefore, a bar graph summary is presented here for comparison. NS9283 reaches maximum potentiation at 3–30 μM. Maximum normalized increases in responses by NS9283 compared with ACh applied alone are 370 ± 22% for $\alpha4\beta2$, 143 ± 10% for $\alpha4\beta4$, and 86 ± 20% for $\alpha2\beta2$. *Error bars* represent the mean ± S.E.

Results

NS9283 Potentiates α2 and α4* but Not α3* nAChRs*—As reported by others, NS9283 augments activation of nAChRs containing three $\alpha2$ or three $\alpha4$ subunits per nAChR expressed in oocytes or HEK cells but not $\alpha3$* nAChRs (10, 12). Here we used nAChRs expressed in HEK cells to validate the pharmacological characteristics of the NS9283 that we synthesized (27). NS9283 increased activation by ACh (at EC_{20-30} concentration) of $\alpha2$* and $\alpha4$* cell lines by 86–371% (Fig. 1). An $\alpha2\beta4$ line was not affected by NS9283. Cell lines may contain mixtures of subtypes, for example $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ in the $\alpha4\beta2$ line and $(\alpha2\beta4)_2\alpha2$ and $(\alpha2\beta4)_2\beta4$ in the $\alpha2\beta4$ line. Subtypes containing sites at which NS9283 can bind (*e.g.* $\alpha4/\alpha4$ in $(\alpha4\beta2)_2\alpha4$ or $\alpha2/\alpha2$ in $(\alpha2\beta4)_2\alpha2$) exhibit increased responses with NS9283, but the $(\alpha2\beta4)_2\beta4$ stoichiometry cannot bind NS9283 (12). We tested potentiation by NS9283 of $(\alpha2\beta4)_2\alpha2$ nAChRs expressed in oocytes (cRNA injection ratio of $\alpha2$ to $\beta4$, 4:1). NS9283 increased activation by 100 μM ACh of $\alpha2\beta4$ nAChR by 268% with an EC_{50} of 5.02 ± 1.81 μM and reached maximum potentiation at 30 μM. This suggests that our unresponsive $\alpha2\beta4$ cell line expresses mainly the $(\alpha2\beta4)_2\beta4$ stoichiometry, whereas the responsive $\alpha4\beta2$, $\alpha4\beta4$, and $\alpha2\beta2$ nAChR cell lines express the $(\alpha\beta)_2\alpha$ stoichiometry and possibly the $(\alpha\beta)_2\beta$ stoichiometry. NS9283 did not potentiate activation by ACh on $\alpha3\beta2$ or $\alpha3\beta4$ cell lines (Fig. 1). NS9283 did not activate any $\alpha2$*, $\alpha3$*, or $\alpha4$* nAChR by itself as reported by others (data not shown) (10, 12).

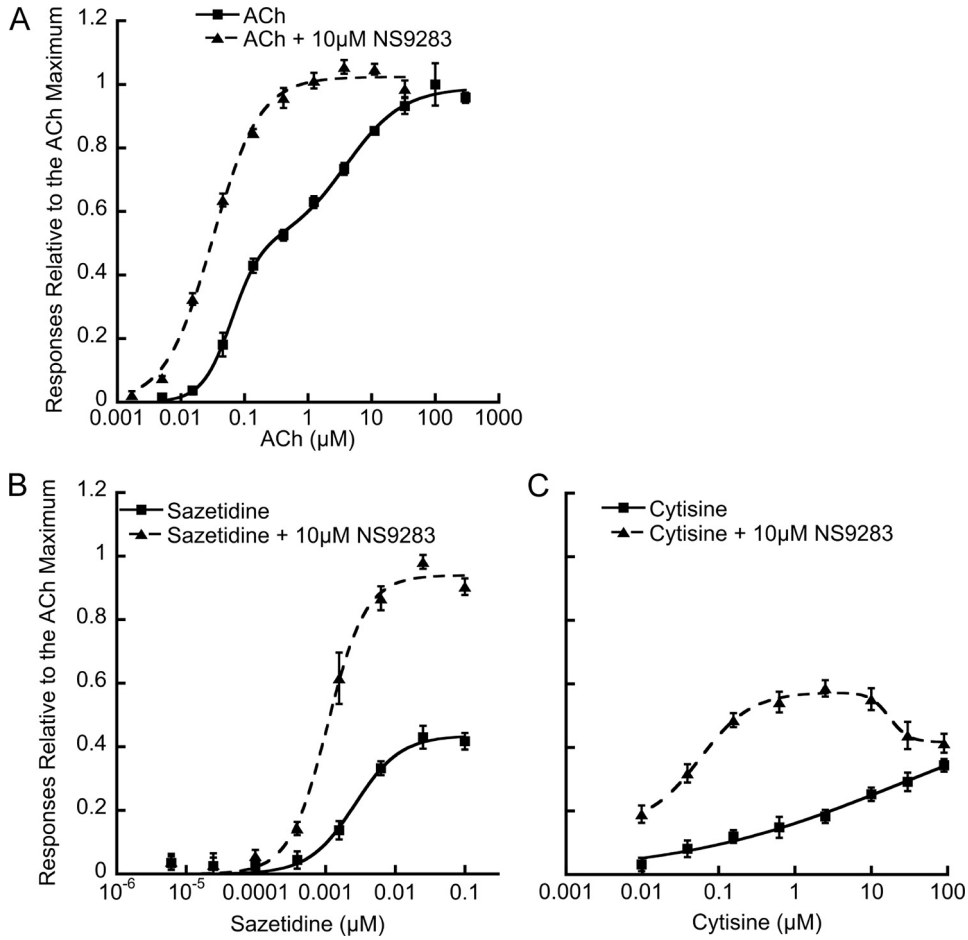

FIGURE 2. **NS9283 potentiates activation of $\alpha4\beta2$ nAChRs by ACh, sazetidine, and cytisine differently.** 10 μM NS9283 was incubated for 15 min with HEK cells stably expressing $\alpha4\beta2$ nAChRs before acute application of various concentrations of sazetidine or cytisine. A fluorescent indicator was used to record the membrane potential changes. *A*, concentration/response curves of ACh with and without NS9283. Note the two-component curve with ACh, reflecting a high affinity contribution from the $\alpha4/\beta2$ sites and a low affinity contribution from the $\alpha4/\alpha4$ site. The efficacy of ACh is 49.9 ± 7.1%, and the EC_{50} is 0.0626 ± 0.0099 μM for the $\alpha4/\beta2$ sites. The efficacy for the low sensitivity $\alpha4/\alpha4$ sites is 49.1 ± 8.5%, and the EC_{50} is 3.89 ± 1.35 μM. After the $\alpha4/\alpha4$ site is occupied by preapplied NS9283, sensitivity reflects acutely applied ACh activating at high affinity $\alpha4/\beta2$ sites. The efficacy of ACh with NS9283 is 102 ± 1%, and the EC_{50} is 0.0314 ± 0.0024 μM. *B*, concentration/response curves of sazetidine with and without NS9283. The efficacy of sazetidine alone is 43.5 ± 2.1%, and the EC_{50} is 0.00262 ± 0.00047 μM. The efficacy of sazetidine with NS9283 is 94.0 ± 2.6%, and the EC_{50} is 0.00107 ± 0.00012 μM. *C*, concentration/response curves of cytisine with and without preapplication of 10 μM NS9283. The maximum efficacy of cytisine alone (≤90 μM) is 34.3 ± 2.0%, and the concentration/response curve does not reach plateau even at 90 μM. The efficacy of cytisine with NS9283 is 57.9 ± 1.9%, and the EC_{50} is 0.0259 ± 0.0044 μM. Responses of 90 μM cytisine with NS9283 plunge to 41.3 ± 3.0% of the maximum responses evoked by ACh. *Error bars* represent the mean ± S.E.

NS9283 Increases Activation by Agonists—Both sazetidine and cytisine are partial agonists for $\alpha4\beta2$ expressed in HEK cells (20). Our $\alpha4\beta2$ cell line expresses a mixture of $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ stoichiometries as illustrated by the biphasic activation curve by ACh alone in Fig. 2*A* (8). Partial agonism of sazetidine arises from its exclusive and high affinity action at the $\alpha4/\beta2$ agonist sites; *i.e.* sazetidine is a primary SSAg (21, 22). Cytisine is an intrinsic partial agonist that non-selectively binds to both the $\alpha4/\beta2$ and $\alpha4/\alpha4$ agonist sites (20). ACh is defined as a full agonist for both the $\alpha4/\alpha4$ site and $\alpha4/\beta2$ site. We investigated whether NS9283, which is an accessory SSAg, assists activation by ACh, sazetidine, and cytisine. At low concentrations of ACh, NS9283 from the $\alpha4/\alpha4$ site increased activation of $\alpha4\beta2$ nAChRs, but at high concentrations of ACh, NS9283 did not exceed the efficacy of ACh (18). This is because NS9283 cannot compete with ACh to bind to the $\alpha4/\alpha4$ sites, or if it does, it is functionally indistinguishable from ACh bound to this site.

NS9283 increases activation of nAChRs by partial agonists, but its potentiation profiles differ between the $\alpha4/\beta2$ primary SSAg sazetidine and the non-selective partial agonist cytisine. NS9283 increased the efficacy of both sazetidine and cytisine (Fig. 2, *B* and *C*). Interestingly, NS9283 made sazetidine a full agonist at $\alpha4\beta2$ nAChRs without changing its potency. This is most likely because NS9283 activates the $\alpha4/\alpha4$ site (the critical aspect of potentiation), a site that is not bound by sazetidine. NS9283 (10 μM) also potentiated activation of $\alpha4\beta2$ nAChRs by cytisine up to 10 μM but not at higher concentrations of cytisine (Fig. 2*C*). Because NS9283 is an agonist at the $\alpha4/\alpha4$ site and cytisine is a partial agonist at both $\alpha4/\beta2$ and $\alpha4/\alpha4$ sites, NS9283 likely augments activation by cytisine at $\alpha4/\beta2$ sites, whereas increasing concentrations of cytisine compete with NS9283 for binding to the $\alpha4/\alpha4$ site, thereby preventing the full agonist effect of NS9283 at this site. Channel block by cytisine could also contribute to reduced responses at high concentrations of cytisine.

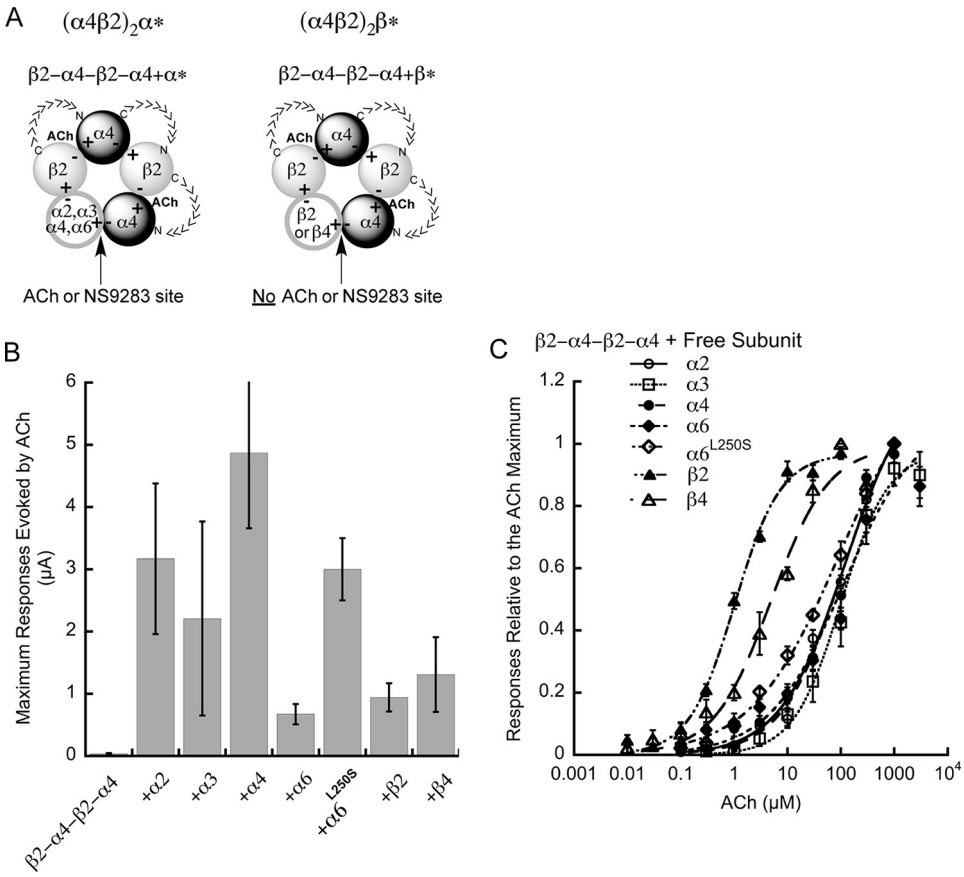

FIGURE 3. **ACh activation of $(\alpha4\beta2)_2$* nAChRs expressed from $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ concatamers and free accessory subunits in oocytes.** *A*, illustrations of nAChRs made from expressing tetrameric concatamer with a free subunit. Accessory subunits are displayed as *open circles*. Structural $\alpha4$ and $\beta2$ subunits are displayed as *solid spheres*. $(AGS)_n$ linkers ($n = 6$ or 12) are illustrated as *arrows* from the C terminus of one subunit to the N terminus of another. ACh sites are located at the interfaces of $\alpha4(+)/\beta2(-)$ or $\alpha(+)/\alpha4(-)$ but not at $\beta(+)/\alpha4(-)$ interfaces. The accessory SSAg NS9283 acts through the $\alpha(+)/\alpha4(-)$ interface. *B*, maximum absolute current of $(\alpha4\beta2)_2$* nAChRs evoked by ACh. *C*, concentration/response curves for ACh activating different $(\alpha4\beta2)_2$* nAChRs. *Error bars* represent the mean ± S.E.

In summary, NS9283, which binds only to the $\alpha4/\alpha4$ site, increases efficacies of SSAgs that bind only to the two $\alpha4/\beta2$ sites and the potencies of agonists that bind to all three sites. Its effect is lost if it is displaced from the $\alpha4/\alpha4$ site by the partial agonist cytisine but not by the full agonist ACh; *i.e.* NS9283 behaves like a full agonist for the $\alpha4/\alpha4$ site.

Activation and Potentiation of $(\alpha4\beta2)_2$ nAChRs with Different Accessory Subunits*—Residues on the minus side of the $\alpha4$ subunit determine agonist sensitivity and selectivity of the $\alpha4/\alpha4$ agonist site (2, 3, 19). To investigate the pharmacological requirement for the plus side of this interface, we expressed $(\alpha4\beta2)_2$* nAChRs with various accessory subunits by injecting oocytes with mRNAs of $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ concatamers and a free accessory subunit (Fig. 3*A*). The concatamer alone gives minimal 300 μM ACh-evoked current, less than 40 nA on average ($n = 17$) (Fig. 3*B*). Concatamer expressed with a free subunit produces maximum ACh-evoked responses ranging from 673 nA to 3.17 μA (Fig. 3*B*). Higher total current and responses dominated by the low affinity agonist site at the $\alpha4/\alpha4$ interface characterize $(\alpha4\beta2)_2\alpha4$ nAChRs (2). Like $\alpha4$, other α structural subunits except $\alpha6$ also show higher absolute currents than β subunits when assembling into the accessory position (Fig. 3*B*). Because of expression variation between individual oocytes, the standard errors of absolute currents are large. Because of assembly, trafficking, and functional issues, it is difficult to express $\alpha6$ in heterologous systems (37). The gain-of-function mutant $\alpha6^{L250S}$ can, however, be more readily expressed with the concatamer $\beta2$-$\alpha4$-$\beta2$-$\alpha4$. This produced an average maximum ACh-evoked current of 3.00 ± 0.50 μA, which is as high as the other α subunits tested. These data suggest that an additional ACh site can form at interfaces of an accessory α with the $\alpha4$ subunits to increase activation. This additional accessory $\alpha6/\alpha4$ site in $(\alpha4\beta2)_2\alpha6^{L250S}$ resulted in 64.8 ± 6.4% inhibition of ACh (300 μM) responses by the $\alpha6$-selective antagonist conotoxin MII (100 nM) (38).

Absolute current differences could be due to different protein expression levels, assembly or maturation efficiencies, and/or channel electrophysiological properties. However, besides absolute current, we observed another characteristic of the agonist site at the accessory $\alpha/\alpha4$ sites, *i.e.* low sensitivity to ACh activation (Fig. 3*C* and Table 1). $\beta2$ and $\beta4$ subunits produced nAChRs highly sensitive to ACh when expressed with $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ with EC_{50} values of 1.02 ± 0.10 μM for $(\alpha4\beta2)_2\beta2$ and 4.96 ± 1.53 μM for $(\alpha4\beta2)_2\beta4$ nAChRs. α subunits ($\alpha2-4$ and $\alpha6$ wild type and mutant) as accessory subunits display low sensitivities to ACh with EC_{50} values higher than 100 μM. Note that $(\alpha4\beta2)_2\alpha$ nAChRs have two high affinity agonist sites at $\alpha4/\beta2$ interfaces. This usually results in ACh concentration/

response curves fitting better to a biphasic curve with a high and a low EC_{50} value (2, 3). Unfortunately, our data set could not resolve the two EC_{50} values very well for all of the nAChR subtypes; thus all dose/response curves were fit to a monophasic curve for comparison. The Hill coefficient values of $(\alpha4\beta2)_2\alpha2$, $(\alpha4\beta2)_2\alpha4$, and $(\alpha4\beta2)_2\alpha6$ calculated in this way are less than 1 (0.625–0.762), consistent with the presence of agonist sites with different affinities.

NS9283 can activate nAChRs into which three $\alpha4/\alpha4$ sites have been engineered (16, 17). However, NS9283 did not activate wild type nAChRs when delivered alone (12) (Fig. 4). We confirm that 10 μM NS9283 selectively potentiates wild type nAChRs through the $\alpha4/\alpha4$ agonist site. 1) It increases activation of $(\alpha4\beta2)_2\alpha4$ nAChRs (*i.e.* $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ + $\alpha4$) by 30 μM ACh (225 ± 43%; Fig. 4, *A* and *B*). 2) It fails to potentiate activation by ACh of $(\alpha4\beta2)_2\beta2$ nAChRs (*i.e.* $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ + $\beta2$; Fig. 4, *A* and *C*). Such potentiation selectivity applies to other α and β subunits. NS9283 increased activation by ACh (30 μM) of $(\alpha4\beta2)_2\alpha2$, $(\alpha4\beta2)_2\alpha3$, and $(\alpha4\beta2)_2\alpha6$ nAChRs by 208–477% but not activation of $(\alpha4\beta2)_2\beta4$ nAChRs (Fig. 4*A*). These data suggest that accessory $\alpha/\alpha4$ interfaces form an agonist site for ACh and NS9283, whereas accessory $\beta/\alpha4$ interfaces do not (Fig. 3*A*).

TABLE 1

Summary of potencies of ACh activation of $(\alpha4\beta2)_2$* nAChRs

$(\alpha4\beta2)_2$* nAChRs were expressed from $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ concatamer and free subunits in oocytes. *n*, number of oocytes tested; N.D., not detected.

Subtypes	EC_{50}	n_H	*n*
	μM		
$\beta2$-$\alpha4$-$\beta2$-$\alpha4$	N.D.	N.D.	17
$\beta2$-$\alpha4$-$\beta2$-$\alpha4$ + $\alpha2$	120 ± 48	0.710 ± 0.098	4
$\beta2$-$\alpha4$-$\beta2$-$\alpha4$ + $\alpha3$	101 ± 20	0.977 ± 0.141	6
$\beta2$-$\alpha4$-$\beta2$-$\alpha4$ + $\alpha4$	108 ± 45	0.762 ± 0.126	10
$\beta2$-$\alpha4$-$\beta2$-$\alpha4$ + $\alpha6$	109 ± 80	0.625 ± 0.166	5
$\beta2$-$\alpha4$-$\beta2$-$\alpha4$ + $\alpha6^{L250S}$	92.7 ± 24.4	0.528 ± 0.032	4
$\beta2$-$\alpha4$-$\beta2$-$\alpha4$ + $\beta2$	1.02 ± 0.10	0.959 ± 0.114	7
$\beta2$-$\alpha4$-$\beta2$-$\alpha4$ + $\beta4$	4.96 ± 1.53	0.801 ± 0.146	5

Selective Blockage of Subunit Interfaces—To enable pharmacological study of accessory sites, we mutated threonine 126 on the minus face of $\alpha4$ to cysteine to allow selective blockage of the agonist binding site at this interface by the thioreactive agent MTSEA (39) (Fig. 5*A*). We also mutated the corresponding amino acid in $\beta2$ subunit, leucine 121, to cysteine to check the efficiency of blocking ACh activation at other subunit interfaces using this mutation and MTSEA. We used ACh at con-

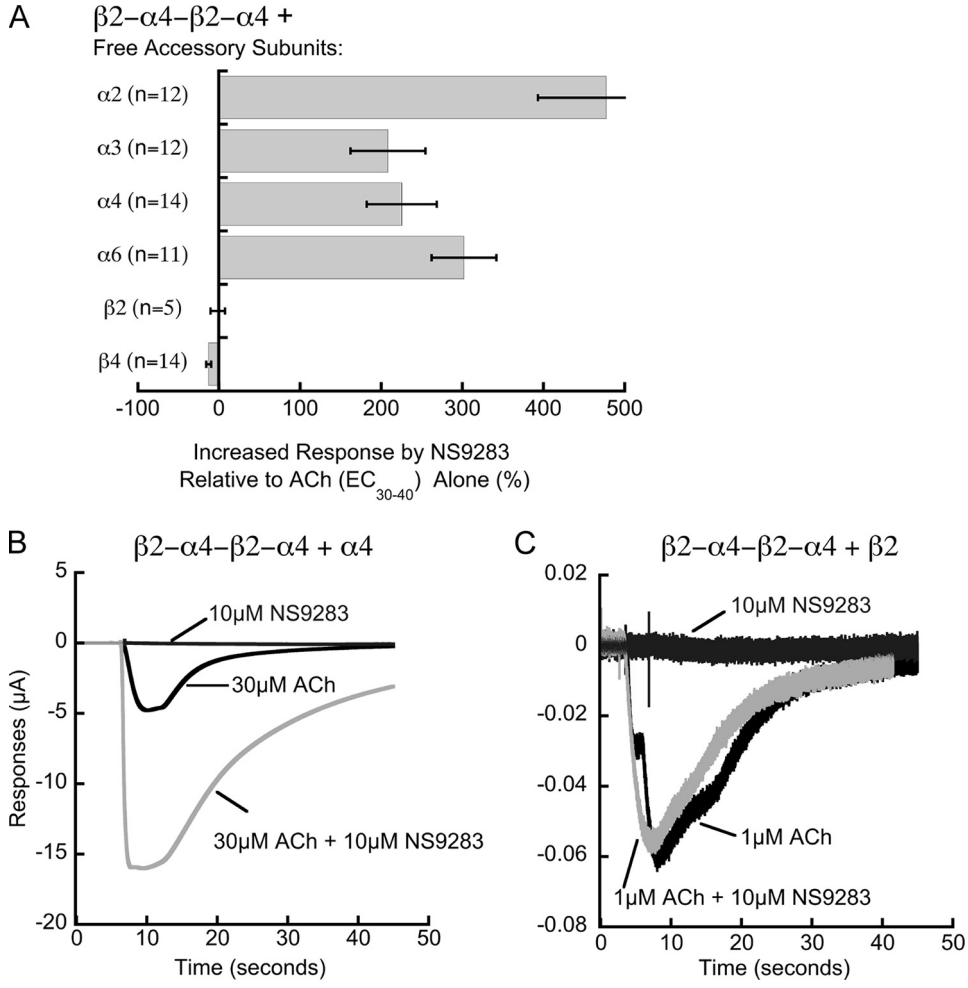

FIGURE 4. **Potentiation of activation of $(\alpha4\beta2)_2$* nAChRs by NS9283.** nAChRs were expressed from $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ concatamers and free accessory subunits in oocytes. 10 μM NS9283 was preapplied to oocytes for 2 min before its co-application with EC_{30-40} concentrations of ACh (*i.e.* 30 μM for all $(\alpha4\beta2)_2\alpha$ nAChRs, 1 μM for $(\alpha4\beta2)_2\beta2$ subtype, and 3 μM for $(\alpha4\beta2)_2\beta4$ subtype). *A*, potentiation of NS9283 was evaluated as increased response relative to ACh applied with vehicle 0.1% DMSO (v/v). NS9283 potentiates activation of $(\alpha4\beta2)_2\alpha$ nAChRs, but not $(\alpha4\beta2)_2\beta$ subtypes, by ACh. *B* and *C*, representative response kinetics for $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ nAChRs. *Error bars* represent the mean ± S.E.

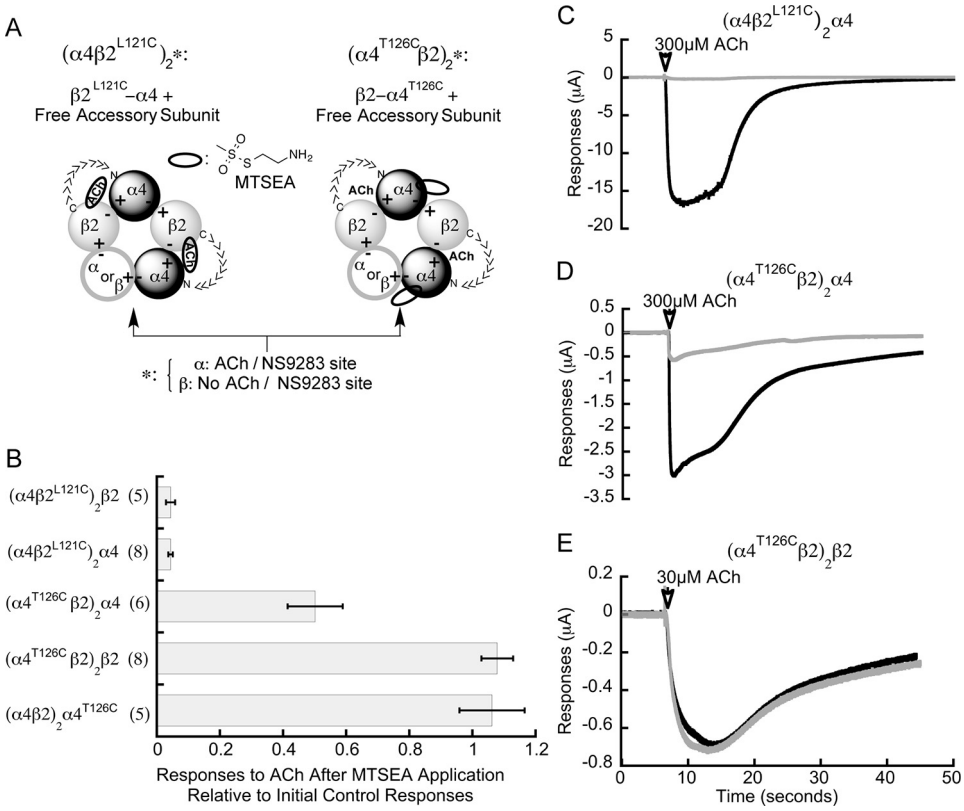

FIGURE 5. **Effects of MTSEA on $\alpha4\beta2$ nAChRs with cysteine mutations engineered at different subunit interfaces.** *A*, schematic illustration of agonist sites and "+" and "−" sides of subunits in nAChRs expressed from $\beta2$-$\alpha4$ concatamer cysteine mutants and free subunits. $(AGS)_n$ linkers ($n = 6$) are illustrated as *arrows* from the C terminus of $\beta2$ to the N terminus of $\alpha4$. Mutations $\alpha4^{T126C}$ and $\beta2^{L121C}$ are at homologous locations on the minus side of these subunits. Reaction of the SH group in cysteine with MTSEA (shown as *open ovals*) forms a disulfide-linked alkyl group blocking binding of ACh or NS9283 if the minus side of the mutated subunit is part of an agonist binding site. Alkylation of cysteines at interfaces like $\beta2/\alpha4$ that do not form an agonist binding sites has no effect. *B*, summary of MTSEA effects on nAChR activation by ACh when blocking different subunit interfaces. Responses of 300 (for concatamer plus $\alpha4$) or 30 μM (for concatamer plus $\beta2$) ACh after MTSEA application are used to evaluate blockage of ACh activation. Values close to 1 indicate no blockage, whereas values less than 1 show blockage of nAChR activation by MTSEA. *C–E*, representative traces. ACh responses from a single oocyte are displayed in *black* (before MTSEA application) or *gray* (after MTSEA application). *Error bars* represent the mean ± S.E.

centrations producing maximal activation to evaluate blockage by MTSEA in the following experiments. Because repeated subunit sequences in the tetrameric concatamer would complicate mutagenesis, we expressed mutated dimeric $\beta2$-$\alpha4$ concatamers with free accessory subunits to allow blockage of different subunit interfaces (Fig. 5*A*). When we engineered such a cysteine mutant at all of the agonist sites such as in $(\alpha4\beta2^{L121C})\beta2$ nAChRs, 2 mM MTSEA abolished activation of these nAChRs by ACh (Fig. 5*B*). Note that, although $(\alpha4\beta2^{L121C})\alpha4$ nAChRs retained an intact agonist site at the accessory $\alpha4/\alpha4$ agonist site after MTSEA treatment, they failed to respond to ACh significantly after MTSEA treatment (Fig. 5, *B* and *C*). This suggests that ACh, like NS9283, cannot activate nAChRs solely from the accessory $\alpha4/\alpha4$ agonist site. This is consistent with the notion that potentiation results from the presence of three rather than two agonist sites (12, 17, 19). We next expressed $(\alpha4^{T126C}\beta2)_2\alpha4$ nAChRs. These nAChRs have intact agonist sites at the $\alpha4/\beta2$ interfaces, a blockable site at the $\alpha4/\alpha4$ interface, and a blockable site at one of the two $\beta2/\alpha4$ interfaces (Fig. 5*A*). MTSEA decreases the activation by 300 μM ACh of these nAChRs by 50 ± 9%. A cysteine mutant at interfaces that do not form an agonist site does not block activation. For example, no blockage by 2 mM MTSEA was observed in $(\alpha4\beta2)_2\alpha4^{T126C}$ or $(\alpha4^{T126C}\beta2)_2\beta2$ nAChRs (Fig. 5). Therefore, the decrease in activation observed for MTSEA-treated $(\alpha4^{T126C}\beta2)_2\alpha4$ probably results from MTSEA preventing activation by agonist from the $\alpha4/\alpha4$ site rather than alkylation at the $\beta2/\alpha4$ interfaces. No MTSEA blockage was observed in wild type nAChRs without MTSEA-reactive sites such as $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ nAChRs (data not shown).

MTSEA Blocks Activation from Many Accessory α/α4 Sites—If an agonist site is present at an accessory site, MTSEA should selectively block this interface of $(\alpha4^{T126C}\beta2)_2$* nAChRs, leaving only the $\alpha4/\beta2$ sites (Fig. 5*A*), *i.e.* decreasing the efficacy and leaving only the high affinity responses. To test this idea, we expressed the mutant concatamer $\beta2$-$\alpha4^{T126C}$ with free subunits in oocytes and investigated the effect of MTSEA on activation by ACh of these nAChRs. Fig. 6 and Table 2 summarize the effects of MTSEA on the potency and efficacy of ACh of these nAChRs.

Like $(\alpha4\beta2)_2\alpha$ nAChRs (Fig. 3*B*), mutant $(\alpha4^{T126C}\beta2)_2\alpha$ nAChRs produced larger total ACh-evoked currents than $(\alpha4^{T126C}\beta2)_2\beta$ nAChRs presumably because of the low affinity agonist site at the accessory $\alpha/\alpha4$ interfaces (Fig. 6 and Table 2). EC_{50} values of ACh activating $(\alpha4^{T126C}\beta2)_2\alpha$ nAChRs range from 21.0 to 37.3 μM, which are lower than the EC_{50} values of wild type $(\alpha4\beta2)_2\alpha$ nAChRs obtained from $\beta2$-$\alpha4$-$\beta2$-$\alpha4$ concatamer (Table 1) but much higher than the EC_{50} values of

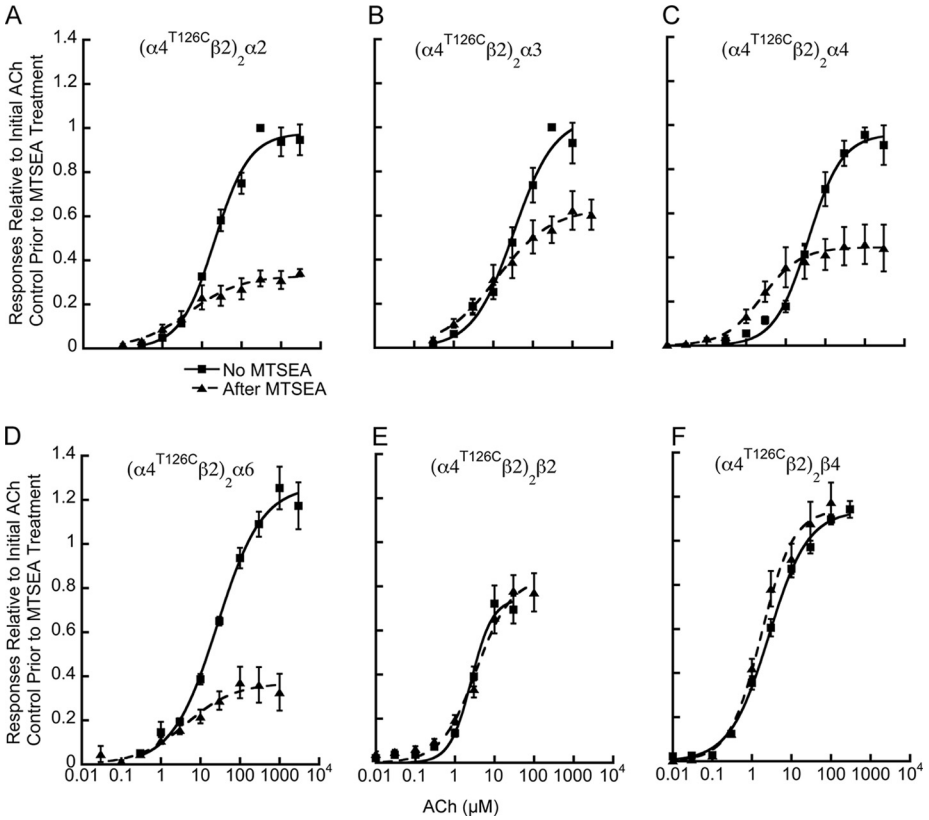

FIGURE 6. **Effects of MTSEA on sensitivity and efficacy of ACh activating $(\alpha4\beta2)_2\alpha$ and $(\alpha4\beta2)_2\beta$ nAChRs.** Mutant concatamer $\beta2$-$\alpha4^{T126C}$ was expressed with free subunit in oocytes to investigate the effect of MTSEA on ACh activating from different accessory interfaces. In all cases, the cysteine is on the minus side of the $\alpha4$ subunit interacting with the plus side of the accessory subunit. *A*, $(\alpha4^{T126C}\beta2)_2\alpha2$; *B*, $(\alpha4^{T126C}\beta2)_2\alpha3$; *C*, $(\alpha4^{T126C}\beta2)_2\alpha4$; *D*, $(\alpha4^{T126C}\beta2)_2\alpha6$; *E*, $(\alpha4^{T126C}\beta2)_2\beta2$; *F*, $(\alpha4^{T126C}\beta2)_2\beta4$. All responses are normalized to the average of the two initial ACh controls (300 μM for $(\alpha4\beta2)_2\alpha$ nAChRs and 30 μM for $(\alpha4\beta2)_2\beta$ nAChRs) without 2 mM MTSEA treatment. *Error bars* represent the mean ± S.E.

TABLE 2

Summary of potencies and efficacies of ACh activating $(\alpha4\beta2)_2$* nAChRs with and without NS9283 before and after MTSEA blockage

$(\alpha4\beta2)_2$* nAChRs were expressed from mutant $\beta2$-$\alpha4^{T126C}$ concatamer and free subunit in oocytes. They have an accessory */$\alpha4^{T126C}$ interface vulnerable to blockage by MTSEA. I_{max}, maximum current normalized to the average of the two initial ACh controls; current, maximum absolute current evoked by ACh; *n*, number of oocytes tested.

Subtype	EC_{50} (μM)	nHill	I_{max}	Current (μA)	n
$(\alpha4^{T126C}\beta2)_2\alpha2$	21.0±3.37	0.992±0.132	0.977±0.034	4.42±1.71	6
with NS9283	0.822±0.150	0.919±0.132	1.03±0.03		7
$(\alpha4^{T126C}\beta2)_2\alpha3$	32.9±11.0	0.841±0.166	1.05±0.09	4.57±1.31	5
with NS9283	1.78±0.45	0.640±0.084	1.02±0.04		8
$(\alpha4^{T126C}\beta2)_2\alpha4$	37.2±4.67	1.02±0.11	0.960±0.028	3.06±0.73	7
with NS9283	0.172±0.039	0.908±0.166	1.17±0.04		5
$(\alpha4^{T126C}\beta2)_2\alpha6$	26.7±4.39	0.764±0.074	1.26±0.04	3.84±1.66	6
with NS9283	2.13±0.38	0.810±0.09	0.987±0.036		7
$(\alpha4^{T126C}\beta2)_2\beta2$	2.59±0.45	1.60±0.38	0.746±0.050	0.512±0.106	6
$(\alpha4^{T126C}\beta2)_2\beta4$	2.68±0.25	0.878±0.057	1.14±0.02	1.94±0.35	8
After MTSEA					
$(\alpha4^{T126C}\beta2)_2\alpha2$	4.76±1.18	0.659±0.089	0.330±0.013		10
with NS9283	0.927±0.098	1.01±0.09	0.401±0.007		7
$(\alpha4^{T126C}\beta2)_2\alpha3$	12.3±2.1	0.628±0.054	0.633±0.019		7
with NS9283	17.7±7.3	0.535±0.069	0.858±0.066		
$(\alpha4^{T126C}\beta2)_2\alpha4$	2.58±0.25	0.902±0.068	0.446±0.007		6
with NS9283	1.78±0.45	0.679±0.094	0.340±0.014		6
$(\alpha4^{T126C}\beta2)_2\alpha6$	4.38±1.54	0.696±0.133	0.369±0.024		4
with NS9283	6.90±1.21	0.591±0.037	0.304±0.011		6
$(\alpha4^{T126C}\beta2)_2\beta2$	3.50±0.86	0.978±0.175	0.841±0.057		8
$(\alpha4^{T126C}\beta2)_2\beta4$	1.71±0.23	1.067±0.128	1.14±0.04		4

$(\alpha4^{T126C}\beta2)_2\beta2$ and $(\alpha4^{T126C}\beta2)_2\beta4$ nAChRs at 2.59 ± 0.45 and 2.68 ± 0.25 μM, respectively. Therefore, although this T126C mutation affects activation by ACh of $(\alpha4\beta2)_2\alpha$, it has retained the additional agonist site at the accessory $\alpha/\alpha4$ sites.

MTSEA attenuated activation by ACh of $(\alpha4^{T126C}\beta2)_2\alpha$ nAChRs (where $\alpha = \alpha2$, $\alpha3$, or $\alpha6$) similarly to the attenuated response in $(\alpha4^{T126C}\beta2)_2\alpha4$ nAChRs. MTSEA reduced maximum ACh-evoked responses of these nAChRs by 42–89% (Figs. 6 and Table 2). After MTSEA blockage of this low sensitivity $\alpha/\alpha4$ ACh site, only the responses of the high sensitivity sites were observed (Fig. 6, *A–D*). The sensitivities to ACh of $(\alpha4^{T126C}\beta2)_2\alpha$ nAChRs after MTSEA blockage are similar to those of $(\alpha4^{T126C}\beta2)_2\beta$ nAChRs (Table 2), suggesting complete blockage of the accessory agonist site in these nAChRs. The EC_{50} value of ACh activating $(\alpha4^{T126C}\beta2)_2\alpha3$ nAChRs after MTSEA blockage is 12.3 ± 2.1 μM, which is 2.7-fold smaller than that before MSTEA blockage but larger than those of $(\alpha4^{T126C}\beta2)_2\beta$ nAChRs. The increase in sensitivity and decrease in efficacy by MSTEA blockage imply that an additional agonist site is present at the accessory $\alpha3/\alpha4$ site (Fig. 6*B*). ACh binding and/or activation of $(\alpha4^{T126C}\beta2)_2\alpha3$ nAChRs is slightly different from that of the other $(\alpha4\beta2)_2\alpha$ nAChRs. Perhaps MTSEA cannot fully block the activation by ACh at this $\alpha3/\alpha4$ interface, or residual low sensitivity activation there partially obscures the responses from high sensitivity sites.

In contrast to the above effects, MTSEA did not affect ACh-induced activation of $(\alpha4^{126C}\beta2)_2\beta$ nAChRs where $\beta = \beta2$ or $\beta4$. MTSEA did not change the maximum ACh-evoked responses of $(\alpha4^{126C}\beta2)_2\beta$ nAChRs (Fig. 5). The EC_{50} values

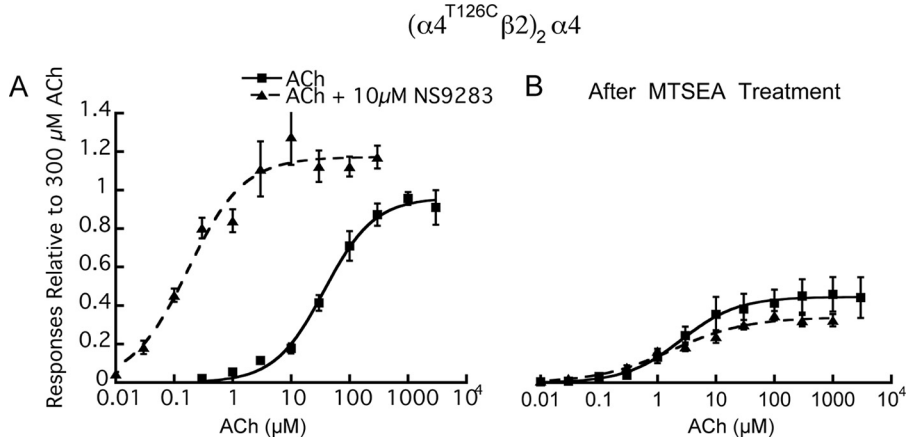

FIGURE 7. **MTSEA abolishes potentiation by NS9283 through the accessory $\alpha4/\alpha4$ agonist site.** 10 μM NS9283 was co-applied with various concentrations of ACh to oocytes expressing mutant dimeric concatamer $\beta2$-$\alpha4^{T126C}$ and $\alpha4$ subunit. These nAChRs have an accessory $\alpha4/\alpha4^{T126C}$ interface vulnerable to blockage by MTSEA. The effect of 2 mM MTSEA on potentiation of NS9283 is compared in *A* and *B*. After MTSEA blockage, NS9283 fails to increase sensitivity to ACh as it does without MTSEA modification. *Error bars* represent the mean ± S.E.

before and after MTSEA treatment are similar for $(\alpha4^{126C}\beta2)_2\beta2$ and $(\alpha4^{126C}\beta2)_2\beta4$ nAChRs (Fig. 6 and Table 2). These data suggest that there are no ACh sites present at accessory $\beta/\alpha4$ interfaces.

NS9283 selectively binds to the accessory $\alpha4/\alpha4$ agonist site, acting as a third agonist to increase the probability of channel opening (16, 17) (Fig. 4). We investigated whether MTSEA blockage at $\alpha4/\alpha4$ and other accessory $\alpha/\alpha4$ agonist sites affects potentiation of $(\alpha4\beta2)_2\alpha$ nAChRs by NS9283. 10 μM NS9283 increases sensitivity to ACh of $(\alpha4^{T126C}\beta2)_2\alpha4$ nAChRs and shifts the EC_{50} of ACh from 37.2 ± 4.67 to 1.78 ± 0.45 μM (Fig. 7*A*). After MTSEA blockage, EC_{50} values of ACh are similar with or without NS9283 at 1.78 ± 0.45 and 2.58 ± 0.26 μM, respectively (Fig. 7*B*). Similarly, MTSEA blocked potentiation by NS9283 on other $(\alpha4^{T126C}\beta2)_2\alpha$ nAChRs (Fig. 8 and Table 2). MTSEA completely abolished the increase of ACh sensitivity on $(\alpha4^{T126C}\beta2)_2\alpha3$ and $(\alpha4^{T126C}\beta2)_2\alpha6$ nAChRs by NS9283 and reduced the increase of ACh potency by NS9283 on $(\alpha4^{T126C}\beta2)_2\alpha2$ nAChRs from 26- to 5.1-fold. These data suggest that NS9283 augments nAChR activation through the accessory $\alpha/\alpha4$ agonist sites as illustrated in Fig. 5*A*; *i.e.* it is an accessory SSAg for many $(\alpha4\beta2)_2\alpha$ nAChRs.

Discussion

There is interest in developing PAM drugs for nAChRs because agonists both activate and desensitize nAChRs (24, 40). PAMs bind away from agonist binding sites to enhance function when agonists are bound. Benzodiazepines are PAMs for γ-aminobutyric acid (GABA) receptors and have proven clinically useful in modulating their function (41). There is optimism that the same principle can be applied to nAChRs. PAMs offer the potential to modulate endogenous patterns of signaling rather than constantly activating or desensitizing as results from sustained exposure to agonist drugs. Here we have defined another group of drugs, accessory SSAgs, such as NS9283, that functionally behave like a PAM but adopt an agonist-like mechanism to potentiate nAChR activation.

NS9283 was understandably designated as a PAM initially because it appeared not to have intrinsic activity at nAChRs or to compete with agonist for binding to nAChRs (9, 10, 12). We confirm that NS9283 does not act as a PAM but instead potentiates activation of nAChRs by binding to a low affinity ACh binding site at the $\alpha4/\alpha4$ subunit interface (17, 19) (Figs. 4, 5*A*, and 7). The low affinity of this site and the site selectivity of NS9283 explain why this compound demonstrated PAM-like activity during its initial characterization (12, 17) (Figs. 1 and 3). Demonstration of agonist activation by NS9283 (16, 17) and identification of selectivity for a particular ACh site (17, 19) (Figs. 3 and 7) disqualify NS9283 as a PAM because it is neither a modulator nor allosteric.

We define NS9283 and other agonists selective for this accessory ACh binding site as accessory SSAgs. NS9283 binds to the extracellular domain of nAChRs (17, 19) differently from PAMs

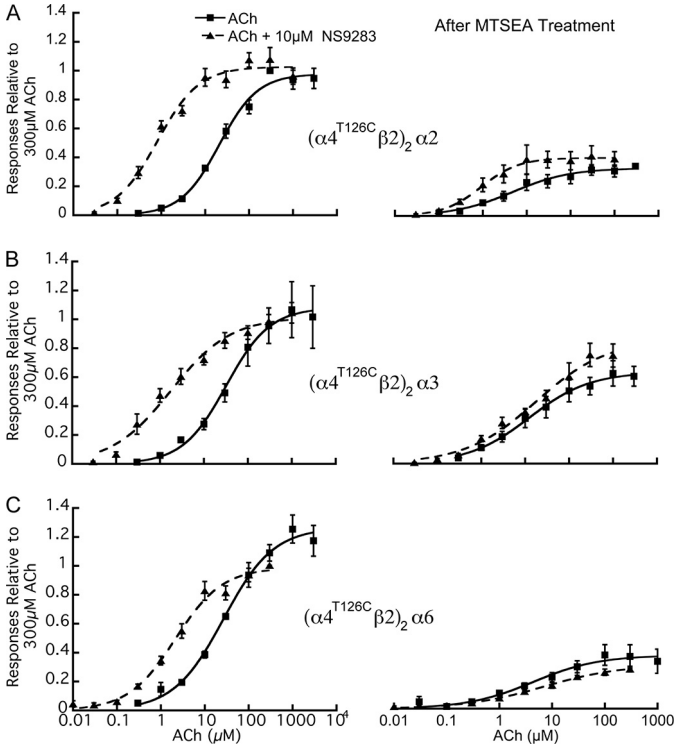

FIGURE 8. **MTSEA abolishes or attenuates potentiation by NS9283 of other $(\alpha4\beta2)_2\alpha$ nAChRs.** *A*, $(\alpha4\beta2)_2\alpha2$ subtype; *B*, $(\alpha4\beta2)_2\alpha3$ subtype; *C*, $(\alpha4\beta2)_2\alpha6$ subtype. *Error bars* represent the mean ± S.E.

and allosteric agonists like PNU-120596 and 4-(4-bromophenyl)-3*a*,4,5,9*b*-tetrahydro-3*H*-cyclopenta[*c*]quinoline-8-sulfonamide, which bind to transmembrane sites near the channel gate whose opening they influence (24–26). Both NS9283 and benzodiazepines bind to the extracellular domain of accessory subunits. However, the accessory SSAg NS9283 binds to the opposite side of the accessory subunit relative to the benzodiazepine PAM site in $GABA_A$ receptors, and the benzodiazepine PAM site is not a GABA binding site (42). Through binding to the $\alpha4/\alpha4$ agonist site, NS9283 cannot activate wild type nAChRs by itself because single agonist site occupancy is not sufficient to efficiently activate nAChRs (6). Neither can ACh activate from binding only to the accessory $\alpha4/\alpha4$ agonist site (Fig. 5*C*). Hence, the accessory agonist site appears to serve as a novel potentiation site that binds agonists and potentiates activity in response to ACh binding at the other agonist sites. Binding only to the accessory site is not sufficient to induce desensitization nor can it prevent or reverse desensitization resulting from agonist binding to $\alpha4/\beta2$ sites (18). However, NS9283 increases sensitivity of ACh desensitization because it occupies the $\alpha4/\alpha4$ site as an agonist (18). Binding only to the accessory site cannot up-regulate nAChRs. Agonists and antagonists at $\alpha4/\beta2$ sites are thought to increase the amount of nAChRs by promoting a conformation of $(\alpha4\beta2)_2$ that assembles more efficiently with accessory subunits to form mature nAChRs (36). A binding site for NS9283 does not exist in the $(\alpha4\beta2)_2$ assembly intermediate. Therefore, NS9283 did not up-regulate $\alpha4\beta2$ nAChRs expressed in HEK cells (data not shown). The potentiation mechanism of accessory SSAgs like NS9283 provides a target mechanism for development of novel therapeutics.

Developing a new Food and Drug Administration-approved drug can take more than 7 years on average to pass through clinical trials, not to mention their high cost, low success rate, and high preclinical expenses (43). Drugs like NS9283 could be cost-effective because they offer unique properties when combined with already approved agonists and partial agonists. Indeed, by enhancing agonist activation and desensitization affinities, such drugs have the potential to reduce side effects of high doses of nAChR agonists such as nausea and motor or cardiovascular impairment, which result from nonspecific interaction with other receptors (9–11). This property could also enhance older drugs to treat new diseases or syndromes by enhancing their activity without the need to increase their dose (10). Our *in vitro* characterization of NS9283 has provided insights in guiding design of combined therapy for SSAgs and other known nAChR agonists. NS9283 increases agonist site occupancy by binding to accessory agonist sites (17) (Figs. 7 and 8), which results in gain of function of both full and partial agonists (Fig. 2). Because of different binding site selectivity, NS9283 binds to the accessory $\alpha4/\alpha4$ site independently from binding of sazetidine to $\alpha4/\beta2$ sites, thus turning this primary SSAg into a full agonist without altering its potency. Conversely, ACh and the partial agonist cytisine bind to both the $\alpha4/\beta2$ and $\alpha4/\alpha4$ agonist sites (20). NS9283 primes the $\alpha4/\alpha4$ agonist sites for these non-selective agonists binding to the $\alpha4/\beta2$ sites but competes with them binding to the $\alpha4/\alpha4$ sites, thus increasing their potencies but retaining partial agonism of cytisine and maximum efficacy of ACh.

$\alpha4\beta2^*$ nAChRs are the most abundant nAChR subtypes in the mammalian brain, accounting for 90% of high affinity nAChRs (44). They assemble with additional subunits *in vivo* and form more complex $\alpha4\beta2^*$ subtypes, including but not limited to $\alpha4\beta2\alpha2^*$, $\alpha4\beta2\alpha3^*$, $\alpha4\beta2\alpha6^*$, and $\alpha4\beta2\beta4$ (37, 45, 46). These nAChRs could form one accessory $\alpha/\alpha4$ agonist site and two identical or different α/β agonist sites, *e.g.* $(\alpha4\beta2)(\alpha2\beta2)\alpha4$ and $(\alpha4\beta2)(\alpha4\beta2)\alpha2$. Like $\alpha4$, other accessory α subunits ($\alpha2$, $\alpha3$, and $\alpha6$) can form binding sites for ACh and NS9283 (Figs. 6, 7, and 8). These $\alpha/\alpha4$ sites have low affinity for ACh (Fig. 3 and Table 1) but contribute greatly to nAChR activation efficacy (Fig. 6 and Table 2). Accessory agonist sites that promote much greater activation of nAChRs could be good drug targets. NS9283, which targets $\alpha/\alpha4$ accessory sites, has proven beneficial in improving cognition and reducing pain in rodents (9–15). Both $\alpha2^*$ and $\alpha6^*$ are more prevalent in primates than rodents (37, 47); thus drugs specifically targeting accessory $\alpha2/\alpha4$ and $\alpha6/\alpha4$ agonist sites could be even more beneficial in humans.

Like ACh, NS9283 binds to the interface between the minus face of $\alpha4$ subunit and plus face of $\alpha2$, $\alpha3$, $\alpha4$, and $\alpha6$ (Fig. 4). These α subunits share a C-loop and several aromatic residues that are critical for agonist binding. In the crystal structure of NS9283 and ACh-binding protein (17), these residues from the plus sides interact with the pyridine ring of NS9283 that is partially protonated under physiological pH and resembles the charged nitrogen of an agonist. Therefore, when MTSEA prevents agonist binding at accessory $\alpha/\alpha4$ interfaces via electric repulsion (39), it can also disrupt potentiation of NS9283 binding to these agonist sites (Figs. 7 and 8).

Here we showed the relationship between activation of the two kinds of ACh binding sites. 1) The $\alpha4/\beta2$ sites are necessary for activation by accessory $\alpha/\alpha4$ sites. 2) The accessory $\alpha/\alpha4$ sites promote activation of nAChRs from $\alpha4/\beta2$ agonist sites. ACh cannot activate $(\alpha4^{L121C}\beta2)\alpha4$ nAChRs after MTSEA blocks their $\alpha4/\beta2$ agonist sites (Fig. 5). This is consistent with the observation that occupying one agonist site does not efficiently activate nAChRs (6). It is possible that MTSEA induces an inactive conformation of nAChRs after reacting with $(\alpha4^{L121C}\beta2)^*$, preventing further activation of nAChRs. However, other ligands selective for the $\alpha4/\alpha4$ sites, such as NS9283, cannot activate wild type nAChRs (Fig. 4), which also supports the low occupancy theory. Therefore, accessory SSAgs like NS9283 that bind only to one site are unlikely to activate nAChRs by themselves and will behave pharmacologically like a PAM *in vivo*. Maximum activation by ACh is about equal to NS9283 with other agonists and much better than cytisine with or without NS9283 because cytisine is a very low efficacy partial agonist at both $\alpha4/\alpha4$ and $\alpha4/\beta2$ agonist sites (Fig. 2). Perhaps that is because the bridged bicyclic ring of cytisine precludes the C-loop from closing properly to fully activate nAChRs (48). A partial agonist structure like cytisine should be avoided in designing accessory SSAgs with high efficacies.

In conclusion, we confirm and extend the mechanism proposed by others (2, 3, 16, 17) that there is a binding site for ACh and NS9283 at many $\alpha/\alpha4$ subunit interfaces that promotes

activation by providing a third agonist site in addition to ACh binding sites at the $\alpha4/\beta2$ subunit interfaces in $(\alpha4\beta2)_2^*$ nAChRs. Low affinity accessory ACh binding sites may not encounter sufficient ACh concentrations to activate them during volume transmission in the brain. Postsynaptic brain nAChRs like those in muscle may transiently be exposed to ACh concentrations sufficiently high to fully activate low affinity $\alpha/\alpha4$ sites. It is unknown whether in the brain different stoichiometry nAChRs such as $(\alpha4\beta2)_2\beta2$ and $(\alpha4\beta2)_2\alpha4$ are co-localized to provide broad ACh concentration/response curves or localized in different circuits to mediate different functions. High affinity accessory SSAgs and site-selective antagonists could help to sort this out.

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