

An Allosteric Modulator of $\alpha 7$ Nicotinic Receptors, *N*-(5-Chloro-2,4-dimethoxyphenyl)-*N'*-(5-methyl-3-isoxazolyl)-urea (PNU-120596), Causes Conformational Changes in the Extracellular Ligand Binding Domain Similar to Those Caused by Acetylcholine^[S]

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Received March 13, 2009; accepted May 1, 2009

ABSTRACT

Nicotinic acetylcholine receptors are implicated in several neuropsychiatric disorders, including nicotine addiction, Alzheimer's, schizophrenia, and depression. Therefore, they represent a critical molecular target for drug development and targeted therapeutic intervention. Understanding the molecular mechanisms by which allosteric modulators enhance activation of these receptors is crucial to the development of new drugs. We used the substituted cysteine accessibility method to study conformational changes induced by the positive allosteric modulator *N*-(5-chloro-2,4-dimethoxyphenyl)-*N'*-(5-methyl-3-isoxazolyl)-urea (PNU-120596) in the extracellular ligand binding domain of $\alpha 7$ nicotinic receptors carrying the L247T mutation. PNU-120596 caused changes in cysteine accessibility at the inner beta sheet, transition zone, and agonist binding site. These changes in accessibility are similar to but not identical to those caused by ACh alone. In particular, PNU-120596 induced

changes in MTSEA accessibility at N170C (in the transition zone) that were substantially different from those evoked by acetylcholine (ACh). We found that PNU-120596 induced changes at position E172C in the absence of allosteric modulation. We identified a cysteine mutation of the agonist binding site (W148C) that exhibited an unexpected phenotype in which PNU-120596 acts as a full agonist. In this mutant, ACh-evoked currents were more sensitive to thiol modification than PNU-evoked currents, suggesting that PNU-120596 does not bind at unoccupied agonist-binding sites. Our results provide evidence that binding sites for PNU-120596 are not in the agonist-binding sites and demonstrate that positive allosteric modulators such as PNU-120596 enhance agonist-evoked gating of nicotinic receptors by eliciting conformational effects that are similar but nonidentical to the gating conformations promoted by ACh.

Nicotinic acetylcholine receptors (nAChRs) are the prototypical member of the Cys-loop family of ligand-gated ion channels that also includes GABA_A, serotonin type 3 (5-HT₃), and glycine receptors. This family of receptors assembles as heteromeric or homomeric pentamers around a central pore

(Karlin, 2002). Each subunit contains an extracellular ligand-binding domain (LBD), an α -helical transmembrane domain (TMD), a transition zone that couples the LBD to the TMD, and an intracellular domain (Gay and Yakel, 2007).

Neuronal nAChRs are expressed diffusely throughout most of the central nervous system; $\alpha 7$ -containing receptors show the highest levels of expression (Orr-Urtreger et al., 1997). Of the neuronal nicotinic receptors, the homomeric $\alpha 7$ receptor is implicated in neurological diseases such as schizophrenia, Alzheimer's Disease, and anxiety disorders (Gotti and Clementi, 2004). Therefore, the $\alpha 7$ nicotinic receptor represents an important therapeutic target.

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grant DA017882].

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.109.056226.

[S] The online version of this article (available at <http://molpharm.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; TMD, transmembrane domain; PAM, positive allosteric modulator; PNU-120596, *N*-(5-chloro-2,4-dimethoxyphenyl)-*N'*-(5-methyl-3-isoxazolyl)-urea; LBD, ligand binding domain; SCAM, substituted cysteine accessibility method; ESLC, extracellular solution, low calcium; MTS, methanethiosulfonate; MTSEA, 2-aminoethylmethanethiosulfonate; ACh, acetylcholine; C-O, closed-open gating transition; WT, wild type.

Over the last several years, there has been success in developing synthetic positive allosteric modulators (PAMs) for $\alpha 7$ nAChRs, including PNU-120596 (Bertrand and Gopalakrishnan, 2007). These compounds are predicted to bind away from the orthosteric agonist binding sites and enhance gating of the receptor in the presence of agonists. PNU-120596 is part of a growing class of PAMs that can reopen $\alpha 7$ receptors from the desensitized state and slow additional desensitization, designated as type II modulators (Grønlien et al., 2007). By eliminating the desensitized state, type II PAMs exert a much greater effect on $\alpha 7$ receptor activation than agonists or PAMs that do not alter desensitization (type I modulators).

In animal models, PNU-120596 can partially restore auditory gating deficits (Hurst et al., 2005), a common symptom of schizophrenia. Understanding the molecular mechanisms and structural determinants of PAM action could lead to the development of drugs for the treatment of a wide variety of neuropsychiatric disorders. For example, structural elements from cytosine and morphine guided the development of varenicline, a $\alpha 4\beta 2$ partial agonist and $\alpha 7$ agonist that reduces drug-seeking behavior and consumption of nicotine (Mihalak et al., 2006).

To understand how different nAChR subtypes contribute to disease states, it is crucial to understand the molecular mechanisms by which these receptors couple the binding of agonists and PAMs to opening of the channel. Benzodiazepines, the archetypal positive allosteric modulators of GABA_A receptors, induce conformational changes in the ligand-binding domain of GABA_A receptors (Sharkey and Czajkowski, 2008). We have found that agonists of $\alpha 7$ receptors induce structural transitions in the LBD, as measured by the substituted cysteine accessibility method (SCAM) (Lyford et al., 2003; McLaughlin et al., 2006, 2007). Based on the existing data, we hypothesize that PAMs and agonists cause similar but nonidentical conformational changes.

Here, we used SCAM to compare changes in cysteine accessibility caused by PNU-120596 and ACh. We found that PNU-120596 induced conformational changes in the inner β sheet, transition zone, and orthosteric site that were similar to but not identical to those induced by ACh. We have identified mutations that either eliminate or enhance allosteric modulation to the point of full agonism. Finally, we present evidence that PNU-120596 does not bind at the agonist-binding site.

Materials and Methods

Reagents. Female *Xenopus laevis* frogs were obtained from Xenopus One (Dexter, MI) or Xenopus Express (Brooksville, FL). Methanethiosulfonate (MTS) chemicals were obtained from Toronto Research Chemicals (Toronto, ON, Canada). PNU-120596 was obtained from Tocris Bioscience (Ellisville, MO). The QuikChange site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA) and the mMessage mMachine in vitro RNA transcription kit was obtained from Ambion (Austin, TX). All other reagents for molecular biology, oocyte dissection, and electrophysiological recordings were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Molecular Biology. The chick $\alpha 7$ nAChR was expressed in the pAMV vector under the control of the T7 promoter. Mutations were introduced into C115A/L247T receptors using the QuikChange site-

directed mutagenesis kit according to the manufacturer's instructions, and were verified by DNA sequencing. All receptors contained a cysteine-to-alanine mutation at position 115. Cys115 is the only unpaired cysteine in the LBD, and the C115A mutation simplifies the interpretation of thiol modification experiments without affecting responses to ACh (McLaughlin et al., 2006) or PNU-120596. The utility of the L247T mutation is described under *Results*. Capped cRNA transcripts were made as described previously (Lyford et al., 2003).

Construct Expression in *X. laevis* oocytes. *X. laevis* oocytes were surgically removed as described previously (Lyford et al., 2003). The oocytes were injected with 20 ng of $\alpha 7$ nAChR cRNA and were incubated for 2 to 7 days in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM Na-HEPES, pH 7.5) plus 50 mg/ml gentamicin and 0.55 mg/ml sodium pyruvate.

Some mutants, displaying a peak current response to maximal ACh of less than 100 nA, were coexpressed with human RIC-3 protein (resistant to inhibitors of cholinesterase) in a 1:1 (w/w) cRNA ratio (Lansdell et al., 2005). Dose-response curves from several mutant $\alpha 7$ receptors were generated with and without coexpression of RIC-3. The data suggest that RIC-3 substantially increased the peak current amplitude without major effects on the ACh EC₅₀ values (Supplemental Table 1).

Two-Electrode Voltage Clamp of *X. laevis* oocytes. Oocytes were superfused with ESLC (96 mM NaCl, 2 mM KCl, 10 mM MgCl₂, 0.1 mM CaCl₂, and 10 mM HEPES-NaOH, pH 7.5), a low-Ca²⁺ solution that minimizes currents through Ca²⁺-activated Cl⁻ channels. For each mutant $\alpha 7$ nAChR, a 5 to 7 point dose-response curve was generated to ACh alone or with the addition of 1 μ M PNU-120596 (in ESLC). Dose responses for ACh with and without PNU-120596 were obtained from the same oocytes. Unless otherwise noted, PNU-120596 was preapplied for 30 s and then coapplied with ACh. For some mutant receptors, a dose-response curve was generated for the allosteric modulator in the presence of an EC₃₀₋₅₀ dose of ACh. All dose response curves were fit to a three-parameter Hill equation using SigmaPlot 9.0 (Systat Software, San Jose, CA). Data were reported as the average \pm S.E.M. Two-electrode voltage clamp was performed as described previously (McLaughlin et al., 2007). Solutions were applied by gravity perfusion with a flow rate of 3 to 5 ml/min. Oocytes were superfused with ESLC for at least 2 min between all drug applications, and current amplitudes returned to baseline.

To eliminate complications of run-up and run-down of current over the course of an experiment, all oocytes were initially treated with a maximal ACh dose 3 to 4 times consecutively. Oocytes were discarded if the response to the maximal ACh dose varied by more than $\pm 10\%$.

Substituted Cysteine Accessibility Method. MTS reagents were made fresh daily in distilled H₂O and stored on ice. Just before use, the MTS reagents were diluted to the appropriate concentration in ESLC and were applied immediately to the oocytes. 2-Aminoethyl methanethiosulfonate (MTSEA) was our test compound to screen for reactivity at introduced thiols. We compared the ACh dose response curves of each mutant before and after application of a high concentration of MTSEA (0.5–10 mM for 0.5–4 min). For mutants that showed a functional effect of MTSEA, a limiting concentration of MTSEA (0.1–100 μ M), yielding 20 to 50% of the maximal MTSEA effect, was determined empirically. To measure modification rates, the limiting concentration of MTSEA was applied repeatedly for 15 to 30 s. In experiments with PNU-120596, 1 μ M was preapplied for 30 s and then coapplied with the limiting concentration of MTSEA. In experiments with agonists, an EC₁₀₀ (a maximally effective concentration) was coapplied with the limiting concentration of MTSEA. In all experimental conditions, the functional effect of MTSEA modification was tested with a \sim EC₅₀ concentration of ACh. Each application of ACh was applied until a peak current amplitude was obtained (~ 15 s), and then the perfusion was switched back to ESLC

(wash buffer). At the end of each experiment, a maximal dose of MTSEA was applied (0.5–10 mM) to measure the effect when all accessible thiols were modified.

Normalized current amplitudes ($(I_t - I_\infty)/(I_{\text{zero}} - I_\infty)$, where I_t is the current amplitude after the cumulative time of MTSEA exposure, I_∞ is the current amplitude after the final maximal dose of MTSEA, and I_{zero} is the initial current amplitude before modification) were fit to a single exponential decay (Pascual and Karlin, 1998) using SigmaPlot 9.0. The pseudo first-order rate constant was determined and was divided by the MTSEA concentration to give the second-order rate constant ($\text{molar}^{-1} \text{seconds}^{-1}$).

Statistical Analysis. Hill equation parameters and second-order rate constants were analyzed by one-way analysis of variance with Tukey's post hoc test (SigmaStat 3.0; Systat Software, San Jose, CA). P values of <0.05 were interpreted to indicate significant differences.

Structural Models of the $\alpha 7$ Nicotinic Receptor. A model of the chick $\alpha 7$ nicotinic receptor extracellular domain, based on the coordinates of the *Lymnea stagnalis* ACh Binding Protein (Brejc et al., 2001) was constructed as described previously (Lyford et al., 2003; McLaughlin et al., 2006). Images of the model were generated with Pymol (DeLano Scientific, South San Francisco, CA). For reference, Leu247 is located in the transmembrane domain, in the pore-lining M2 helix, approximately a third of the pore's length from the intracellular end (Revah et al., 1991; Unwin, 2005).

Results

Effect of Mutations on Positive Allosteric Modulation by PNU-120596. Figure 1 shows our homology model of the $\alpha 7$ nAChR derived from the structure of the *L. stagnalis* Acetylcholine Binding Protein (Brejc et al., 2001) and the regions of interest targeted in this study. Trp148 and Trp54 are located in the agonist-binding "pocket" (the orthosteric site). Met37, Asn52, and Met40 are located at the interface between subunits (the inner β sheet), "below" the agonist binding pocket. These residues were previously shown to be good reporters of agonist-induced conformational changes (McLaughlin et al., 2007). Glu44, Asn170, and Glu172 are located in the "transition zone" that couples the LBD to the TMD.

All mutants in this study contained the well characterized leucine 247-to-threonine (L247T or L9'T) mutation. L247T-containing $\alpha 7$ receptors have conductance and ion selectivity that are similar to those of the wild-type receptor but are more sensitive to acetylcholine and exhibit slower macroscopic desensitization (Revah et al., 1991). L247T is a good model system for our studies, because its large current amplitudes allow us to measure modification rates for cysteine

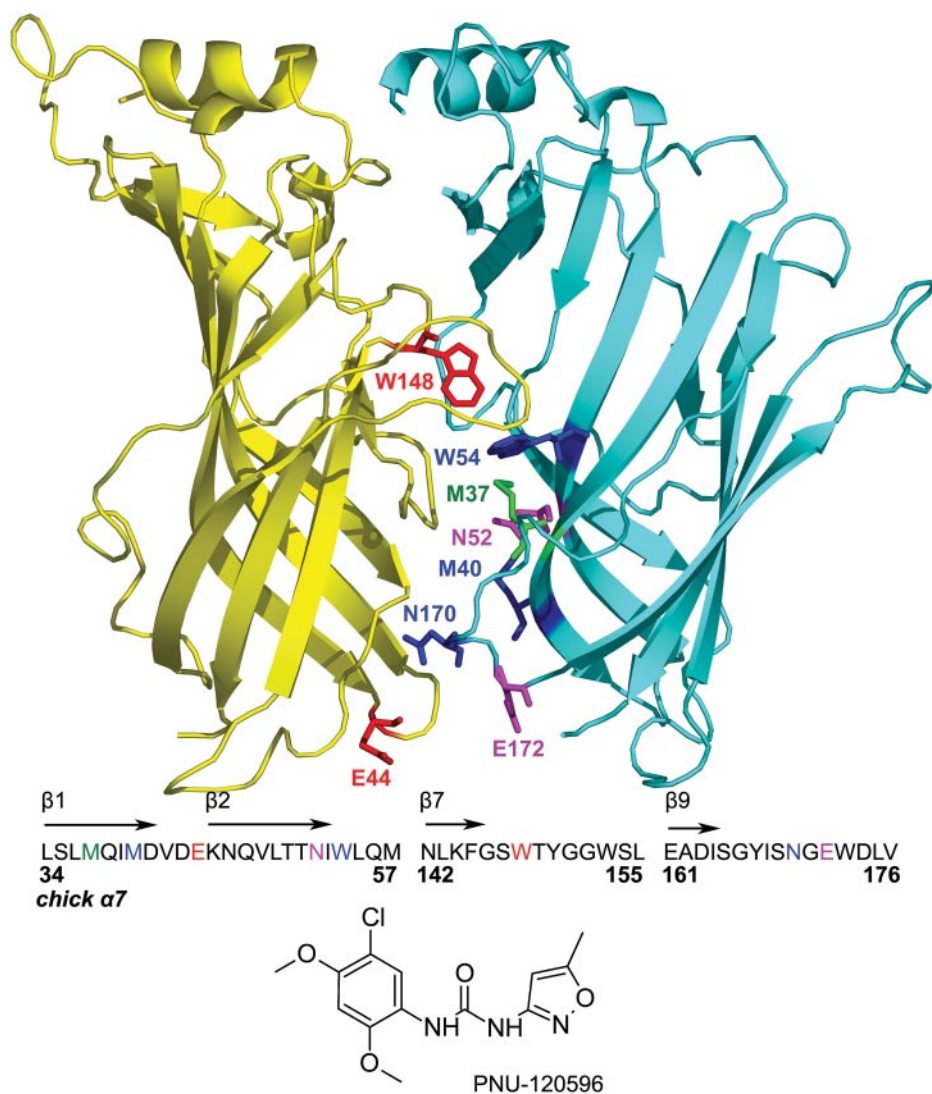


Fig. 1. Homology model of the extracellular domain of $\alpha 7$ nAChR. A ribbon cartoon displaying two subunits of the pentameric receptor, viewed from the outside. The primary ACh-binding subunit is shown in yellow and the complementary subunit is in cyan. Residues of interest are shown as sticks and are labeled on one of the two subunits for clarity. Met37, Met40, and Asn52 are part of the inner β sheet ($\beta 1$, $\beta 2$, $\beta 6$). Glu44, Asn170, and Glu172 are part of the transition zone (loops 2 and 9). Trp54 and Trp148 are part of the orthosteric (agonist-binding) site ($\beta 6$, and behind the C loop). The residues of interest and the surrounding amino acid sequence is shown beneath the cartoon. The structure of PNU-120596 is shown below the chick $\alpha 7$ nAChR amino acid sequence.

substitutions with decreased functional expression levels. All mutants also contained the C115A mutation, in which the single unpaired cysteine in the LBD is mutated to an alanine. This mutation, which has no effect on activation kinetics, ligand sensitivity, or ion permeation, simplifies interpretation of cysteine modification experiments (McLaughlin et al., 2006).

We first examined whether PNU-120596 acts as a positive allosteric modulator of C115A/L247T $\alpha 7$ receptors. PNU-120596 retained its modulatory effect in the C115A/L247T background, as measured by the ability to enhance ACh-evoked currents in a dose-dependent manner (Fig. 2A). The concentration of PNU-120596 that elicited a half-maximal modulation (when applied with an EC_{30-50} concentration of ACh) was 257 ± 22 nM (Fig. 2A), a value that is similar to that reported for wild-type $\alpha 7$ receptors (216 ± 64 nM; Hurst et al., 2005). This suggests that the C115A/L247T mutations do not significantly alter the affinity of PNU-120596 for the $\alpha 7$ nicotinic receptor.

PNU-120596 ($1 \mu\text{M}$) caused a left-shift of the ACh dose-response curve of C115A/L247T $\alpha 7$ receptors and a significant decrease in the EC_{50} for ACh (Fig. 2B, Table 1). In contrast to the responses of wild-type $\alpha 7$ receptors (Hurst et al., 2005), PNU-120596 did not cause a significant change in the current amplitudes of C115A/L247T $\alpha 7$ receptors evoked at maximal ACh concentrations. We speculate that the L247T mutation increased the "gating constant" of the receptors (Colquhoun, 1998), lowering the ACh EC_{50} compared with wild-type receptors. For receptors with a low gating constant, such as wild-type $\alpha 7$ receptors, a PAM could increase the maximal response, decrease the EC_{50} , or both. In contrast, for receptors with a high gating constant, such as L247T-containing $\alpha 7$ receptors, we expect PAMs to affect EC_{50} alone, because the maximal response to agonist is already near 1 (Colquhoun, 1998).

A unique feature of PNU-120596 is the ability to reactivate desensitized $\alpha 7$ receptors in the presence of agonist, a feature defined as type II modulation (Hurst et al., 2005; Grønlien et al., 2007). Therefore, we determined whether PNU-120596 could reactivate C115A/L247T $\alpha 7$ receptors after slow desensitization. A representative trace is shown in Fig. 2C. As expected, C115A/L247T receptors showed partial desensitization during continuous application of an EC_{100} concentration of ACh ($100 \mu\text{M}$, a maximally effective concentration), reaching a plateau at $48.2 \pm 0.02\%$ desensitization by 87.5 ± 7.0 s ($n = 5$). Then, application of an EC_{100} concentration of PNU-120596 ($1 \mu\text{M}$) after partial desensitization reactivated the C115A/L247T receptors ($n = 5$), consistent with type II modulation. The continuous application of ACh and PNU-120596 completely blocked slow desensitization of C115A/L247T receptors, as observed in wild-type $\alpha 7$ receptors (Grønlien et al., 2007). The ability of PNU-120596 to reactivate partially desensitized C115A/L247T receptors and prevent subsequent desensitization did not depend on the ACh concentration (data not shown). We conclude that the C115A/L247T mutations do not alter the affinity or kinetics of PNU-120596 (Fig. 2, A and C), only the ability to enhance peak current amplitude (Fig. 2B), which we attribute to enhanced gating of the C115A/L247T receptors. This interpretation is supported by macroscopic and single-channel analysis, showing that effects on apparent desensitization by mutations at L9' in other Cys-loop receptors can be explained by increases

in mean open time alone (Filatov and White, 1995; Bianchi and Macdonald, 2001). Therefore, $\alpha 7$ receptors containing the C115A/L247T mutation are a reasonable model to examine conformational transitions underlying allosteric modulation by PNU-120596.

For all of the cysteine mutations used in this study, we generated ACh concentration-response curves to probe for possible deleterious effects of the individually introduced cysteines on channel function. Most cysteine mutations gen-

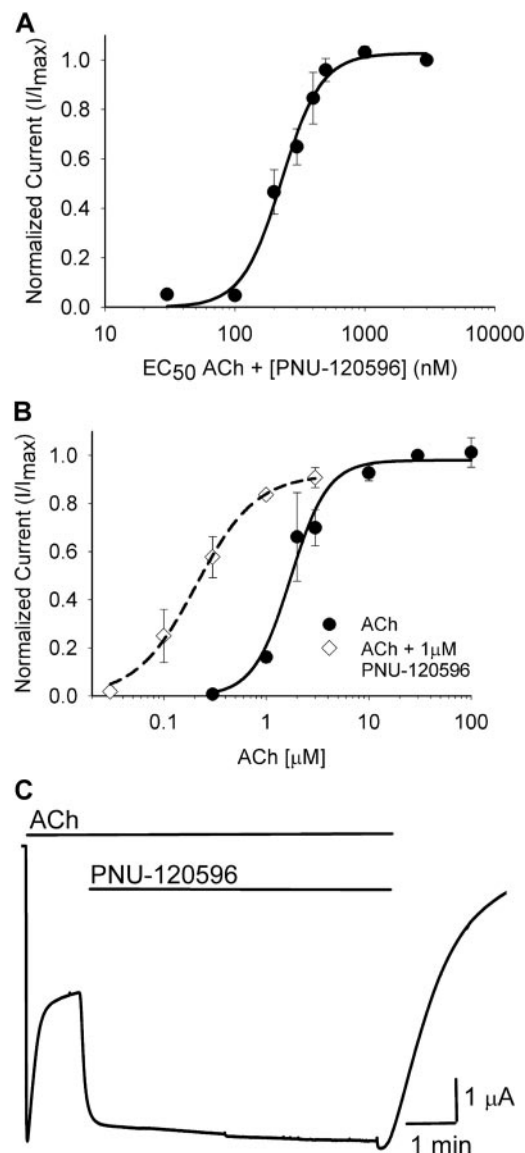


Fig. 2. PNU-120596 is a positive allosteric modulator of C115A/L247T $\alpha 7$ receptor and elicits opening from a partially desensitized state. A, PNU-120596, at the concentrations shown, was applied with an $\sim EC_{50}$ dose of ACh ($2\text{--}3 \mu\text{M}$). Positive allosteric modulation was observed as a significant enhancement of ACh-evoked current ($189 \pm 18\%$, $n = 5$). The EC_{50} for PNU-120596 was 257 ± 22 nM ($n = 5$). B, ACh was applied with an EC_{100} dose of PNU-120596 ($1 \mu\text{M}$). Positive allosteric modulation was observed as a significant reduction in the EC_{50} for ACh (Table 1). Data are fit to the Hill equation and are the mean value \pm S.E.M, normalized to the maximal value of the Hill equation fit of each data set. C, a representative trace showing that an EC_{100} dose of PNU-120596 ($1 \mu\text{M}$) is sufficient to reactivate partially desensitized C115A/L247T receptors ($n = 5$). Coapplication of ACh and PNU-120596 is also sufficient to completely inhibit desensitization, and responses are reversible when both compounds are washed out.

It is important to note that SCAM measures the time-averaged conformational transition of the receptor, including closed, open, desensitized, and multiple intermediate states. Because the L9'T mutation increases the open time of $\alpha 7$

Using this protocol, we determined the second-order rate constants for modification of three residues in the inner β sheet (M37C, M40C, N52C). Fig. 4A shows the mean second-order rate constants measured in the presence of MTSEA alone (control), MTSEA plus ACh, and MTSEA plus PNU-120596. PNU-120596 and ACh each caused a 7- to 9-fold reduction in the modification rate of M37C and a 5- to 7-fold reduction in the modification rate of M40C (Fig. 4B). The modification rates in the presence of ACh or PNU-120596 were significantly different from control but were not significantly different from each other. Thus, without activating these receptors, PNU-120596 caused changes in the accessibility or electrostatic environment of M37C and M40C that were similar to those caused by ACh (McLaughlin et al., 2007). PNU-120596 decreased the rate of modification of M37C, even though receptors containing this mutation were not positively modulated by PNU-120596 (Table 1). This result demonstrates that PNU-120596 can elicit conformational changes in the inner β sheet in the absence of a modulatory effect. Because Met37 or Met40 are not part of the agonist binding site, it is unlikely that steric interference between ligand and MTSEA is responsible for the decreased

Mutant	ACh EC ₅₀	I_{\max}	Hill Coefficient	n	ACh EC ₅₀ + 1 μ M PNU-120596	n	Effect ^a
	μ M	μ A			μ M		
C115A/L247T background	2.4 \pm 0.23	6.4 \pm 0.64	2.8 \pm 0.25	10	0.22 \pm 0.09	3	PAM
Inner β sheet							
M37C	4.5 \pm 1.30	0.4 \pm 0.12	1.3 \pm 0.10	10	1.6 \pm 0.18	7	N.E.
M40C	6.7 \pm 0.70	2.2 \pm 0.47	1.4 \pm 0.11	16	0.58 \pm 0.17	4	PAM
N52C	0.79 \pm 0.20	0.31 \pm 0.08	0.68 \pm 0.07	13	N.D. ^b		
Transition zone							
E44C	7.2 \pm 0.56	4.6 \pm 0.39	2.6 \pm 0.31	10	2.5 \pm 0.37	3	PAM
N170C ^c	12 \pm 1.4	1.3 \pm 0.24	1.6 \pm 0.12	15	2.6 \pm 0.56	3	PAM
E172C ^c	30 \pm 2.0	0.74 \pm 0.11	2.4 \pm 0.10	16	41 \pm 5.3	3	N.E.
Agonist-binding site							
W54C	88 \pm 9.7	2.3 \pm 0.40	1.6 \pm 0.04	12	15 \pm 5.1	5	PAM
W148C ^c	205 \pm 28	3.5 \pm 0.52	1.5 \pm 0.06	11	N.D. ^b		

^c Coexpression with human RIC-3 (see *Materials and Methods*).

modification rates observed at these positions. In contrast, PNU-120596 did not cause a difference in the rate of modification of N52C (Fig. 4, A and B). Although, ACh increased the rate of MTSEA modification of N52C (McLaughlin et al., 2007), PNU-120596 did not cause a significant change. Thus, PNU-120596 caused some but not all of the changes in thiol accessibility in the inner β -sheet caused by ACh. To verify that the conformational changes studied in L247T $\alpha 7$ receptors were similar to those in wild-type $\alpha 7$ receptors (WT), we also studied cysteine accessibility of M40C constructed in the WT background. The rates of MTSEA modification of M40C/WT, with and without ACh, were similar to those measured

in the C115A/L247T background (Supplemental Fig. 1). This suggests that the changes in accessibility that we measure are independent of the C115A/L247T mutation.

Next, we determined the second order rate constants for the modification of three residues in the transition zone (E44C, N170C, and E172C). Fig. 5A shows the mean second-order rate constants measured in the presence of MTSEA alone (control), MTSEA plus ACh, and MTSEA plus PNU-120596. We observed differential effects of PNU-120596 in all three mutant receptors. PNU-120596 and ACh each decreased the rate of MTSEA modification of E44C by ~ 8 - and ~ 21 -fold, respectively (Fig. 5, A and B). PNU-120596 and ACh both decreased the rate of modification of E172C by 2- to 5-fold, even though receptors containing this mutation were not positively modulated by PNU-120596 (Table 1). This result demonstrates that PNU-120596 can elicit conformational changes in the transition zone in the absence of a modulatory effect. The rate of MTSEA modification of N170C was altered differently by PNU-120596 and ACh. PNU-120596 significantly decreased the rate of modification, whereas ACh increased the rate of modification. Overall, these data show that PNU-120596 induces conformational changes at Glu44 and Glu172 that are similar to those induced by ACh, but PNU-120596-induced changes at Asn170 are different from those induced by ACh.

We also measured the combined effects of ACh plus PNU-120596 on MTSEA modification at introduced cysteines in the transition zone (E44C, N170C, and E172C). At E44C and E172C, the combination of PNU-120596 and ACh caused a larger effect than that caused by either ACh or PNU-120596 alone (Fig. 5, Table 2). At N170C, PNU-120596 and ACh had opposite effects on the rate of MTSEA modification. The simultaneous application of both reagents gave a rate of MTSEA modification that was not significantly different from control. In this respect, combined effects of PNU-120596 and ACh led to a net cancellation of each individual effect.

Because the vestibule of the LBD of nicotinic receptors is predicted to be highly electronegative (Unwin, 2005), we also examined the rates of MTS modification using anionic MTS reagents (Supplemental Data Figs. 2–4). We found that MTSEA modified E44C much faster than MTSCE and MTSES (Supplemental Data Fig. 3), even after accounting for the lower intrinsic rates of reaction by MTSCE and MTSES (Supplemental Fig. 2). In addition, the effect of ACh on the rate of MTSEA modification was much greater than the effect of ACh on the rate of MTSCE modification. Modification of M40C by either MTSES or MTSCE was too slow to measure (Supplemental Fig. 4). These results provide additional evidence for a highly negative electrostatic environment lining the vestibule of AChRs and suggest that changes in the electrostatic environment affect modification rates at residues in the inner β sheet and transition zone.

Fig. 6 shows second-order rate constants of MTSEA modification of two residues in the orthosteric agonist binding site (W54C and W148C). ACh slowed the rate of modification of W54C, which agrees with previously published results on wild-type $\alpha 7$ receptors (Gay et al., 2008). However, PNU-120596 did not significantly reduce the rate of modification at W54C (Fig. 6A). ACh and PNU-120596, acting as agonists (Table 1), both significantly slowed the rate of modification of W148C (Fig. 6A). Because the aromatic side chains of Trp54 and Trp148 are both known to be part

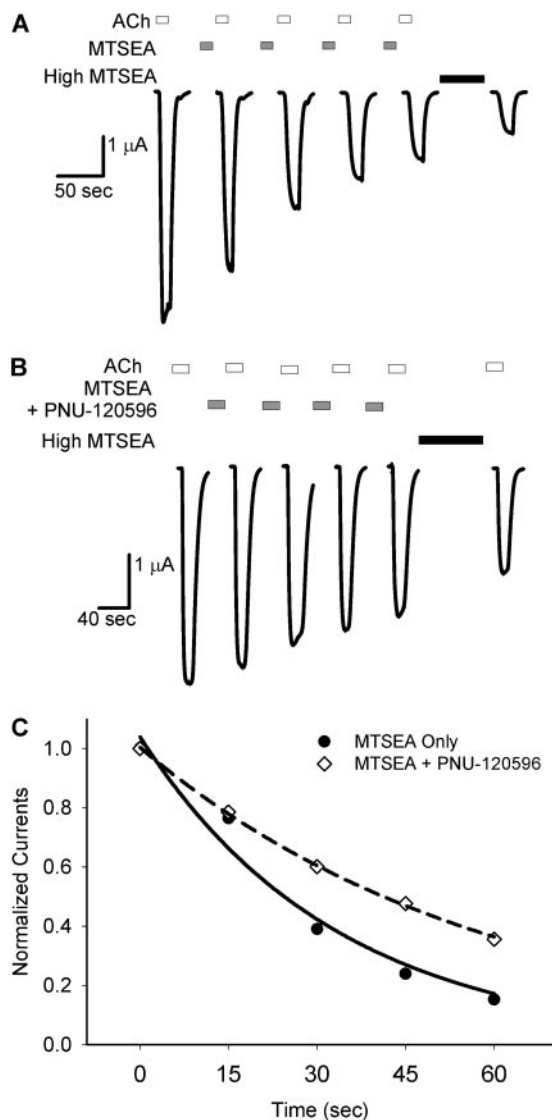


Fig. 3. PNU-120596 slows the rate of MTSEA modification at E44C. A, successive ACh-evoked currents before and after the addition of a sub-maximal concentration of MTSEA (1 μ M, 15-s exposures). After four successive applications, the endpoint of MTSEA modification was determined by a prolonged application of 500 μ M MTSEA for 60 s. Washes of 1 to 2 min between drug applications are not shown. B, the same protocol as A, with a 30-s pre-exposure to 1 μ M PNU-120596 followed by coapplication of 1 μ M PNU-120596 with MTSEA. C, normalized peak current amplitudes from a single experiment are plotted against cumulative MTSEA exposure. The calculated pseudo-first-order rate constants from these experiments are 0.0299 s^{-1} for control (A), and 0.0169 s^{-1} in the presence of PNU-120596 (B). Second-order rate constants (k_2) are calculated from these values and are displayed in Table 2.

of the ligand-binding site, it is likely that steric occlusion by ACh is at least partially responsible for the slowing of modification of these Cys mutants. Slowing of modification of W148C by PNU-120596 could also be explained by steric hindrance, if PNU-120596 binds at unoccupied agonist binding sites, analogous to the binding site for benzodiazepines at the α - γ subunit interface of GABA_A receptors (Günther et al., 1995; Amin et al., 1997). Acetylcholine (M_r 146) contains a positively charged choline group that makes π -cation interactions with the agonist-binding site (Zhong et al., 1998). PNU-120596 (M_r 312) is a urea analog flanked by isoxazole and chlorodimethoxyphenyl groups (Hurst et al., 2005). Recent evidence, however, suggests that PAMs of $\alpha 7$ receptors bind to sites in the TMD (Bertrand et al., 2008; Young et al., 2008), and thus slowing of W148C modification by PNU-120596, could be explained by an allosteric effect at the ligand-binding pocket, perhaps including partial closure of loop C.

To distinguish between these possibilities we took advantage of an unexpected observation: introduction of W148C in the C115A/L247T parent receptor converted PNU-120596 from a positive allosteric modulator to a full agonist (Table 1 and Fig. 7A). The phenotype of this receptor is useful because it allows us to compare the effects of covalent modification with MTS reagents on either ACh- or PNU-120596-evoked gating at the well defined agonist-binding site. If PNU-120596 activates the receptors via binding to the orthosteric agonist binding site, we expect that covalent modification of W148C in the agonist binding pocket to disrupt activation by both ACh and PNU-120596 similarly via steric effects on ligand binding. If PNU-120596 does not activate W148C mutants by binding at the agonist binding site, then PNU-evoked currents should be less sensitive to covalent modification of W148C than ACh-evoked currents. For these experiments, we used two MTS reagents of different size and charge to determine moiety-dependent affects on ACh and PNU-evoked current at W148C. MTSEA adds a positively charged ethyl amine to thiols at physiological pH and is comparatively small. MTSEA-biotin adds a neutral, bulky ring structure to thiols and is comparatively large.

Fig. 7B shows representative ACh- and PNU-120596-evoked currents before and after modification of W148C by MTSEA (100 μ M for 60 s). This exposure to MTSEA was sufficient to completely eliminate ACh-evoked current, but it

had no effect on currents evoked by PNU-120596. If we assume that modification of a binding site residue interferes with activation by steric interference with ligand, then this result implies that PNU-120596 activates receptors via a site distinct from the orthosteric site. However, an alternative explanation is that the two ligands interact with a different set of "contact points" in the binding pocket, and those for PNU-120596 are removed from the ethyl amine adduct at Trp148. To test this, we examined the effect of MTSEA-biotin, which modifies the thiol with a bulky ring structure. Fig. 7C shows representative ACh- and PNU-120596-evoked currents before and after modification of W148C by MTSEA-biotin (4 μ M for 60 s). This exposure of MTSEA-biotin was sufficient to reduce ACh-evoked current by 70% but had no significant effect on the magnitude of currents evoked by PNU-120596. Modification of the agonist binding site by MTSEA-biotin slowed the kinetics of PNU-120596-dependent activation (Fig. 7C), suggesting that there are allosteric conformational changes at the agonist binding site during activation by PNU-120596. The results from multiple experiments are summarized in Fig. 7D. Covalent modification of W148C by MTSEA and MTSEA-biotin had a significantly greater effect on ACh-evoked current than on PNU-120596-evoked currents ($P < 0.01$). This observation provides evidence that PNU-120596, although behaving as an agonist, does not interact with the agonist-binding site of $\alpha 7$ receptors. Our interpretation is that PNU-120596 induces conformational changes at the agonist-binding site through allosteric mechanisms, rather than steric occlusion, because occlusion is predicted to impair both ACh and PNU-evoked currents similarly.

Discussion

In this study, we provide evidence that the positive allosteric modulator PNU-120596 causes conformational changes in the LBD of $\alpha 7$ nicotinic receptors that partially overlap with those caused by ACh. We focused on mapping the structural transitions of PAMs in three regions of the LBD: 1) The inner β sheet, 2) the transition zone, and 3) the orthosteric site (Fig. 1). A homology model of the LBD of the $\alpha 7$ receptor, based on the structure of ACh binding protein, was used to guide our experiments (Brejc et al., 2001; Lyford et al., 2003).

The inner β sheet, composed of the $\beta 1$, $\beta 2$, and $\beta 6$ strands,

TABLE 2

Second-order rate constants for MTSEA modification of receptors carrying Cys substitutions in the extracellular domain of $\alpha 7$ nAChR. Numbers in parentheses represent n .

Mutant	Control	MTSEA + ACh	MTSEA + PNU-120596	MTSEA + ACh + PNU-120596
	$M^{-1}s^{-1}$			
Inner β sheet				
M37C	2470 \pm 310 (12)	333 \pm 152* (6)	271 \pm 104* (7)	
M40C	16,100 \pm 2370 (15)	2090 \pm 748* (5)	3030 \pm 313* (5)	
N52C	250 \pm 68 (8)	1320 \pm 238* (9)	390 \pm 118† (4)	
Transition zone				
E44C	33,700 \pm 6540 (8)	1460 \pm 505* (8)	4180 \pm 637* (5)	312 \pm 19* (5)
N170C	3160 \pm 392 (7)	5730 \pm 682* (7)	818 \pm 114*† (10)	3660 \pm 115 (4)
E172C	12,900 \pm 1560 (9)	3450 \pm 470* (6)	2830 \pm 410* (7)	1710 \pm 310* (7)
Agonist-binding site				
W54C	9500 \pm 1330 (6)	4030 \pm 770* (5)	6080 \pm 920 (4)	
W148C	45,900 \pm 7640 (9)	3010 \pm 1100* (6)	8830 \pm 1320* (6)	

* Statistically different from control ($P < 0.05$).

† Statistically different from + ACh ($P < 0.05$).

resides at the interface between two subunits (Fig. 1). In this study, we found that PNU-120596 caused reductions in the rate of modification of M37C and M40C that were similar to those caused by ACh (Fig. 4, Table 2; McLaughlin et al., 2007). The similarity in the effects on cysteine modification at M37C and M40C suggests that PAMs and agonists induce similar conformational changes in the LBD of $\alpha 7$ receptors. We have provided the first evidence that PAMs of $\alpha 7$ receptors enhance gating by causing some of the same structural transitions in the LBD as ACh.

The transition zone, composed of loops from the LBD (loop 2, loop 9, and the Cys loop) and the TMD (pre-M1 sequence and M2-M3 linker), is positioned to convey structural rearrangements caused by agonist binding in the LBD to the channel gate in the TMD (Bouzat et al., 2004). Therefore, we examined the effect of PNU-120596 on the rate of MTSEA modification at three reporter residues within the transition zone (E44C, N170C, and E172C), which is also a proposed site of modulation by divalent cations (Galzi et al., 1996). PNU-120596 reduced the rate of MTSEA modification at all

three positions (Fig. 5, Table 2). At N170C, PNU-120596 decreased the accessibility of the substituted cysteine, whereas ACh caused an increase. The application of both compounds offset each other at N170C. Overall, the changes in cysteine accessibility in the transition zone caused by ACh and PNU-120596 were similar but not identical.

It is noteworthy that positive modulation of ACh-evoked currents by PNU-120596 was lost in the E172C mutant (Table 1), but PNU-120596 still caused a decrease in thiol accessibility at this position (Fig. 5). One explanation is that the PNU-120596 binding site includes Glu172, and the introduced cysteine (E172C) eliminates allosteric modulation but not binding. In this scenario, the observed reduction in the rate of MTSEA modification at E172C is due to physical occlusion by PNU-120596 of the substituted cysteine at the putative binding site. An alternative explanation is that PNU-120596 binds to a site outside of the transition zone, away from Glu172, and the observed reduction in the rate of E172C modification is due to conformational changes induced there. In this scenario, the binding of PNU-120596 is unaffected and can still induce conformational changes, but the electrostatic coupling within the transition zone (Xiu et

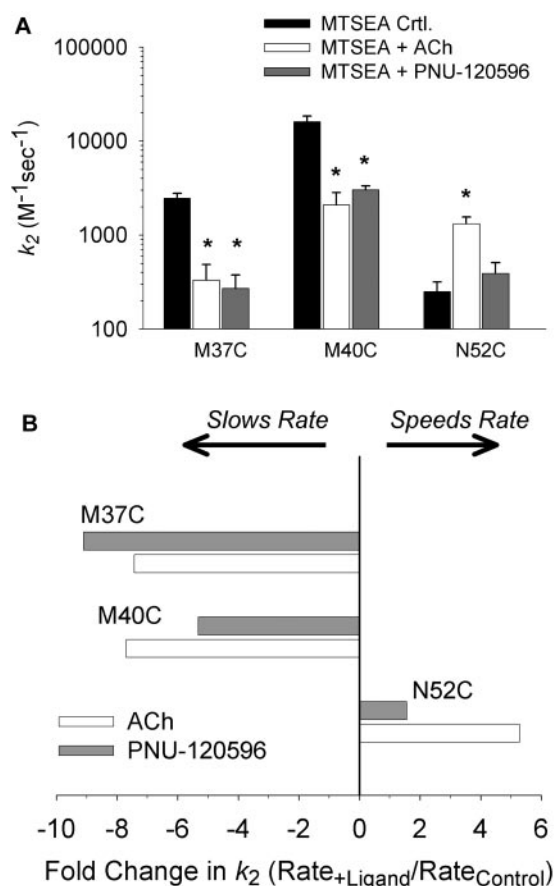


Fig. 4. PNU-120596 differentially alters the rate of MTSEA modification at inner β sheet residues. A, mean second-order modification rate constants (k_2), calculated using the protocol outlined in Fig. 3, at three residues in the inner β sheet, M37C, M40C, and N52C. Mean values for MTSEA alone (control), MTSEA plus ACh, and MTSEA plus PNU-120596 are shown, plotted on a log scale. PNU-120596 slows the rate of modification at M37C and M40C compared with control but has no effect at N52C. *, rate was significantly different from control ($P < 0.05$). B, a plot of the second-order rate constant ratios. The average rate from each experimental condition is divided by the average control rate for each residue. Positive values represent an acceleration of the rate of modification, and negative values represent a reduction in the rate of modification.

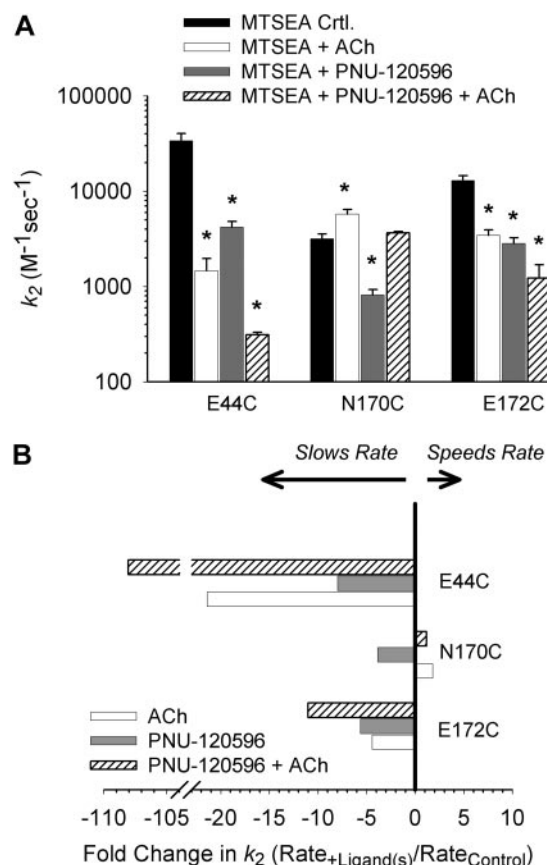


Fig. 5. PNU-120596 differentially alters the rate of MTSEA modification at residues in the transition zone. A, mean second-order modification rate constants (k_2) of at three residues in the transition zone, E44C, N170C, and E172C. PNU-120596 significantly reduces the rate of modification at E44C and N170C. PNU-120596 significantly reduces the rate of modification at E172C, despite the lack of positive allosteric modulation at this residue (Table 1). At N170C, the rate of modification in the presence of PNU-120596 is significantly slower than in the presence of ACh. *, rate was significantly different from control ($P < 0.05$). B, a plot of the second-order rate constant ratios. Note that PNU-120596 slows the rate of modification of N170C whereas ACh increases the rate of modification.

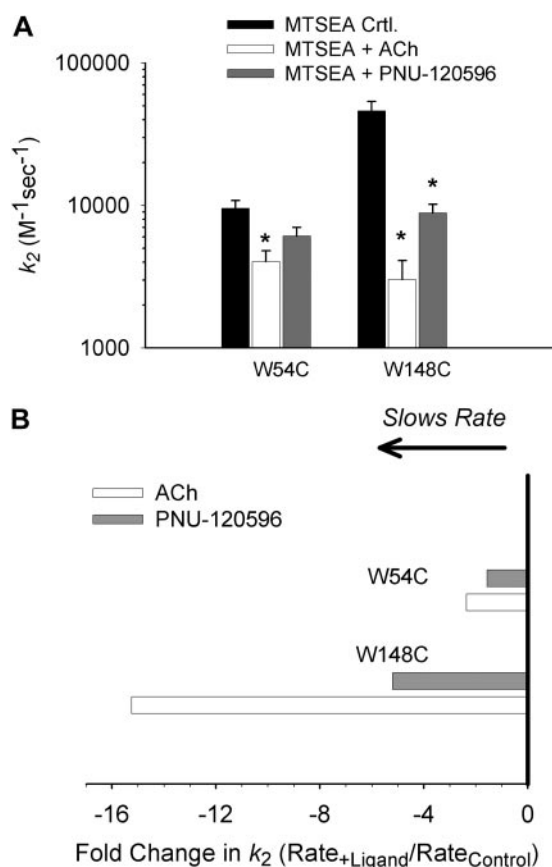


Fig. 6. PNU-120596 slows the rate of MTSEA modification at W148C but not W54C in the orthosteric site. **A**, mean second-order rate constants (k_2) were calculated at two residues in the transition zone, W54C and W148C. *, rate was significantly different from control ($P < 0.05$). **B**, a plot of the second-order rate constant ratios.

al., 2005) is sufficiently disrupted by the cysteine mutation that the induced conformational changes no longer enhance receptor gating.

Finally, we examined the effect of ACh and PNU-120596 on the rate of MTSEA modification at two residues in the orthosteric site (Trp54 and Trp148) (Fig. 6). Viewed from a structural perspective, these residues occupy two distinct locations within the agonist binding site. Trp148 is part of the principal subunit (Fig. 1, yellow subunit). It lines the back wall of the agonist binding site and makes contact with agonists and competitive antagonists (Celie et al., 2004; Hansen et al., 2005). Trp54 is part of the complementary subunit and sits on the edge of the agonist binding site (Fig. 1, cyan subunit) (Brejc et al., 2001). ACh reduced the rate of MTSEA modification at these positions. In our interpretation, ACh reduces covalent modification at W148C by physically blocking access to the introduced cysteine. On the other hand, because carbamylcholine does not contact Trp54 (Celie et al., 2004), we hypothesize that ACh induces a short-range conformational change that makes W54C less accessible to covalent modification. The ACh-dependent effect at W54C agrees with previously published work on wild-type $\alpha 7$ receptors (Gay et al., 2008). PNU-120596 does not significantly affect the rate of MTSEA modification at W54C, but reduced the rate of MTSEA modification at W148C, suggesting that PNU-120596 induces allosteric conformational changes in the center of the agonist binding pocket, but not on the periphery.

We found unexpectedly that PNU-120596 was a full agonist of W148C receptor in C115A/L247T-containing $\alpha 7$ receptors (Fig. 7A). One explanation is that the introduced cysteine allowed partial closure of the C-loop and lowered the activation energy sufficiently to allow PNU-120596 to activate the receptor and at the same time increased energetic barriers to ACh binding. The C-loop is a dynamic and flexible region that acts as a hinge of the orthosteric site (Hansen et

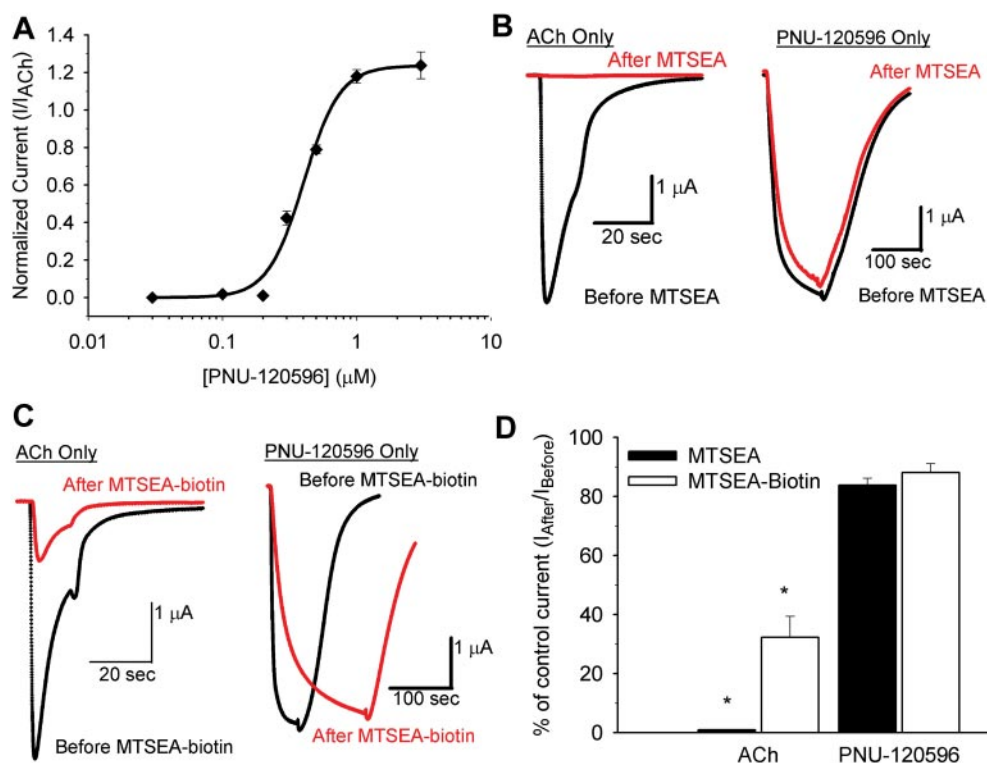


Fig. 7. Covalent modification of W148C in the orthosteric site does not affect agonism by PNU-120596. **A**, PNU-120596 acts as a full agonist of W148C/C115A/L247T $\alpha 7$ receptors. Hill parameter fits for activation by ACh are in Table 1. For PNU-120596, the EC_{50} is $0.45 \pm 0.01 \mu M$, I_{max} is $3.87 \pm 0.65 \mu A$, and Hill coefficient = 6.79 ± 0.81 ($n = 3$). PNU-evoked currents are normalized to peak ACh-evoked current (I/I_{ACh}). **B**, representative traces for 3 mM ACh and 3 μM PNU-120596 shown before (black) and after (red) application of 100 μM MTSEA for 60 s. **C**, representative traces for 3 mM ACh and 3 μM PNU-120596 shown before (black) and after (red) application of 4 μM MTSEA-biotin for 60 s. **D**, summary data for the effects of covalent modification of W148C with MTSEA ($n = 8$) and MTSEA-biotin ($n = 10$) on ACh and PNU-evoked currents. Bars represent the percentage of ACh or PNU-evoked current after MTS modification relative to the control currents before modification (I_{after}/I_{before}). Both MTSEA and MTSEA-biotin caused a significantly greater reduction in ACh-evoked currents than PNU-evoked currents (* $P < 0.01$).

al., 2005; Venkatachalan and Czajkowski, 2008). The EC₅₀ for ACh-dependent activation of the W148C mutant receptor was increased ~100-fold (Table 1), as expected for a receptor with a mutation of an important aromatic residue of the agonist-binding pocket (Brejc et al., 2001). We took advantage of this phenotype to test whether PNU-120596's ability to alter cysteine accessibility was due to allostery or steric occlusion. Because ACh-evoked currents are more sensitive to covalent modification by different MTS reagents than PNU-evoked currents (Fig. 7), we conclude that PNU-120596 induces conformational changes at this position through an allosteric mechanism. A chemically related PAM, NS1738, does not affect equilibrium binding of ¹²⁵I- α -bungarotoxin, also suggesting that it does not interact with the agonist binding site (Timmermann et al., 2007). Recent work with chimeric $\alpha 7$ nAChR/5-HT₃ receptors and mutagenic studies suggests a binding site in the transmembrane domain for PNU-120596 and LY-2087101 (Bertrand et al., 2008; Young et al., 2008). These data suggest that PNU-120596 and other modulators of $\alpha 7$ receptors bind at a conserved site within the transmembrane domain and cause conformational changes in the LBD to enhance gating of the receptor.

In the Monod/Wyman/Changeux model of allostery, positive allosteric modulators of ligand-gated ion channels enhance activation by stabilizing the protein in the open state (Bertrand and Gopalakrishnan, 2007). Our results provide the first evidence that that PNU-120596 promotes activation of $\alpha 7$ receptors by causing some (but not all) of the same conformational changes in the LBD associated with agonists. We have previously shown that permeable divalent cations, which do not alter desensitization, also induce conformational changes in the LBD that are similar to those induced by agonists (McLaughlin et al., 2009). Our work adds to a growing body of literature of both convergent and divergent conformational changes during gating of Cys-loop receptors (Chang and Weiss, 2002; Pless et al., 2007; Sharkey and Czajkowski, 2008; Zhang et al., 2009).

Recent work suggests that the mechanisms of closed-to-open conformational changes induced by different agonists and of unliganded receptors are completely conserved regardless of the agonist applied; only the kinetics of the C-O transitions are affected (Purohit and Auerbach, 2009). Although most of our data with PNU-120596 agrees with this idea, modulation and rate measurements for the N170C mutant seem to be an exception. For this mutant, PNU-120596 slows the rate of modification, whereas ACh increases it (Fig. 5). Thus, each ligand stabilizes a population of conformational intermediates that are distinct from each other and from the resting unliganded state(s). A model in which C-O transitions occur via a single pathway would predict that PNU-120596 and ACh would cause similar changes in accessibility at all residues. If there is a single path, then the observation that PNU-120596 causes a change in accessibility of N170C opposite to that caused by ACh suggests that the receptor would be stabilized in closed intermediate states further away from the open state, and PNU-120596 would be unable to enhance the conserved C-O transition. But PNU-120596 still acts as a positive allosteric modulator of N170C receptors, suggesting that PNU-120596 induces conformational changes along alternative pathways that lower energetic barriers to activation and lead to positive modulation. The differences between the conformational intermediates

induced by PNU-120596 and those induced by ACh are likely to be subtle, because they are not as apparent at other cysteine mutants.

In conclusion, we have shown that PNU-120596 and ACh induce a set of overlapping structural transitions in the extracellular ligand-binding domain. Our results indicate that PAMs such as PNU-120596 enhance gating of ligand-gated ion channels by inducing some of the same structural transitions caused by agonists. In addition, we have identified mutations in the transition zone that eliminate modulation of $\alpha 7$ nicotinic receptors by PNU-120596 via decoupling between the LBD and TMD. We have provided evidence that PNU-120596 does not bind to unoccupied agonist-binding site(s). The PAM-induced changes in receptor kinetics, although not usually sufficient to activate receptors, would lower the energy barriers to agonist-induced activation by both enhancing the agonist-evoked C-O transitions as well as alternate transitions. This process would "prime" the receptors to undergo a gating transition, allowing more of the energy of agonist binding to drive changes in the conformational equilibrium toward activation (Jackson, 1989).

Acknowledgments

We thank Drs. William Green and Millet Treinin for the human RIC-3 clone.

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