

Investigation of the Molecular Mechanism of the $\alpha 7$ Nicotinic Acetylcholine Receptor Positive Allosteric Modulator PNU-120596 Provides Evidence for Two Distinct Desensitized States

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ABSTRACT

Although $\alpha 7$ nicotinic acetylcholine receptors are considered potentially important therapeutic targets, the development of selective agonists has been stymied by the $\alpha 7$ receptor's intrinsically low probability of opening (P_{open}) and the concern that an agonist-based therapeutic approach would disrupt endogenous cholinergic function. Development of $\alpha 7$ positive allosteric modulators (PAMs) holds promise of avoiding both issues. *N*-(5-Chloro-2,4-dimethoxyphenyl)-*N'*-(5-methyl-3-isoxazolyl)-urea (PNU-120596) is one of the most effective $\alpha 7$ PAMs, with a mechanism associated, at least in part, with the destabilization of desensitized states. We studied the mechanism of PNU-120596 potentiation of $\alpha 7$ receptors expressed in *Xenopus laevis* oocytes and outside-out patches from BOSC 23 cells. We identify two forms of $\alpha 7$ desensitization: one is destabilized by PNU-120596

(D_s), and the other is induced by strong episodes of activation and is stable in the presence of the PAM (D_i). Our characterization of prolonged bursts of single-channel currents that occur with PNU-120596 provide a remarkable contrast to the behavior of the channels in the absence of the PAM. Individual channels that avoid the D_i state show a 100,000-fold increase in P_{open} compared with receptors in the nonpotentiated state. In the presence of PNU-120596, balance between D_s and D_i is dynamically regulated by both agonist and PAM binding, with maximal ion channel activity at intermediate levels of binding to both classes of sites. In the presence of high agonist concentrations, competitive antagonists may have the effect of shifting the balance in favor of D_s and increasing ion channel currents.

Introduction

In the absence of agonist, ligand-gated ion channels are primarily in a resting, nonconducting state. With channels that mediate synaptic transmission when the resting condition is perturbed by a sudden elevation in agonist concentration, ligand-binding sites become saturated and the energy landscape for transitions among the various conformational states of the receptor initially favors channel opening, producing periods of synchronous channel activation. Although the equilibrium between activated and nonconducting desen-

sitized states may initially favor activation, desensitization occurs during all phases of a response, and in the prolonged presence of high concentrations of agonist, the ligand-bound receptors accumulate in the states with the lowest free energy; typically, these are nonconducting desensitized states. This general behavior can be ascribed to all nicotinic acetylcholine receptor (nAChR) subtypes, but with some important differences between heteromeric and homomeric ($\alpha 7$) nAChRs.

Heteromeric nAChR such those found at the neuromuscular junction and autonomic ganglia contain two agonist binding sites (Millar and Gotti, 2009). They may open with a single agonist bound, but such openings are usually brief (Colquhoun and Sakmann, 1985) unless the single binding sites remain bound to agonist for prolonged periods (Williams et al., 2011a). The binding of agonist to both sites of a het-

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; PAM, positive allosteric modulator; P_{open} , probability of channel opening; 5HI, 5-hydroxyindole; PNU-120596, *N*-(5-chloro-2,4-dimethoxyphenyl)-*N'*-(5-methyl-3-isoxazolyl)-urea; D_i , type II PAM insensitive desensitization; D_s , type I PAM sensitive desensitization; ACh, acetylcholine; BTMPS, bis-(2,2,6,6-tetramethyl-4-piperidyl) sebacate; TM2, second transmembrane domain; MLA, methyllycaconitine; DH β E, dihydro- β -erythroidine; TQS, 4-(5-(4-chlorophenyl)-2-methyl-3-propionyl-1H-pyrrol-1-yl)benzenesulfonamide; A-867744, 4-(5-(4-chlorophenyl)-2-methyl-3-propionyl-1H-pyrrol-1-yl)benzenesulfonamide; PNU-282987, *N*-(3*R*)-1-azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide.

eromeric receptor has a cooperative effect, increasing the probability of opening (P_{open}) and also promoting the stability of a long-lived open state (Williams et al., 2011a). Homomeric $\alpha 7$ nAChR also activate with submaximal agonist occupancy (Williams et al., 2011a), and the sort of positive cooperativity seen with heteromeric receptors does not seem to occur among the five putative agonist binding sites (Palma et al., 1996). Rapid saturation of the agonist binding sites on $\alpha 7$ receptors may increase the synchronization of whatever activation occurs, but net P_{open} is not increased through cooperative mechanisms, because saturating concentrations of agonist promote a form of rapid desensitization unique to homomeric $\alpha 7$ nAChR (Papke et al., 2000). The unique desensitization of $\alpha 7$ nAChR can be induced rapidly, but it is also readily reversible after the removal of agonist (Papke et al., 2000).

Heteromeric receptors also desensitize rapidly, even at relatively low concentrations of agonist. However, they achieve an equilibrium between activation and desensitization that makes it possible to record prolonged macroscopic responses over a wide range of agonist concentrations (Papke, 2010). The desensitization of heteromeric nAChR does not usually reverse rapidly because these receptors bind agonist with very high affinity in the desensitized state (Heidmann and Changeux, 1978). Modeling these differences, we identify several key features that distinguish the energy landscapes for the conformational transitions of $\alpha 7$ from heteromeric nAChR, including higher energy barriers for entering the open state, low open-state stability, a unique desensitized state, and relatively small energy differences between the resting and desensitized states.

Although the intrinsically low P_{open} of $\alpha 7$ nAChR has presented a special challenge to the study of $\alpha 7$ activation at the level of single-channel currents, new tools have been developed in the form of positive allosteric modulators (PAMs) (Williams et al., 2011b). A number of chemically diverse PAMs have been identified and broadly classified into two groups based on their functional modulation of $\alpha 7$ -mediated responses. The PAMs classified as type I, such as 5-hydroxyindole (5HI), reduce the height of energy barriers for transitions into active states, creating a transient increase in P_{open} immediately after agonist applications but without altering the absolute energy differences between the states, resulting in potentiated responses with conserved kinetics. In contrast, type II PAMs, such as *N*-(5-chloro-2,4-dimethoxyphenyl)-*N'*-(5-methyl-3-isoxazolyl)-urea (PNU-120596), not only increase the magnitude of responses immediately after agonist applications, but also alter the equilibrium between active and desensitized states resulting in dramatically prolonged responses, even promoting activation of previously desensitized receptors (Grønlien et al., 2007).

We have investigated the activation and desensitization of $\alpha 7$ nAChR at the level of macroscopic and single-channel currents, in the presence and absence of PNU-120596. We identify two forms of $\alpha 7$ desensitization. One is sensitive to and destabilized by PNU-120596 (D_s), and the other is insensitive (D_i) to the PAM and, in fact, is promoted by high levels of PNU-120596 binding and potentiated activation. We hypothesize that D_s is associated with the rapid form of desensitization unique to $\alpha 7$, and that in the presence of type II modulators, D_s is connected to an open-state homologous to the long-lived open state observed in diliganded heteromeric

nAChRs. We determine that, even in the presence of PNU-120596, optimal P_{open} may occur at levels of submaximal occupancy of the agonist and PAM binding sites, conditions that minimize stabilization of D_i .

Materials and Methods

cDNA Clones Used for Heterologous Expression in *Xenopus laevis* Oocytes and BOSC 23 Cells. Human nAChR receptor clones and concatamers were obtained from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). The human RIC-3 clone was obtained from Dr. Millet Treinin (Hebrew University, Jerusalem Israel) and coexpressed with $\alpha 7$ to improve the levels and speed of receptor expression (Halevi et al., 2002). The red fluorescent protein clone pDsRed-N1 was obtained from Clontech (Palo Alto, CA) and used to identify successfully transfected BOSC 23 cells. The human $\alpha 7$ T6'S mutant was described previously (Placzek et al., 2005). The L9'T mutation, first characterized in chicken $\alpha 7$ (Bertrand et al., 1997) was inserted into the human cDNA using standard site-directed mutagenesis procedures with a QuikChange kit from Agilent Technologies (Santa Clara, CA) according to the manufacturer's instructions. The mutation was confirmed with automated fluorescent sequencing.

Heterologous Expression of nAChR in *X. laevis* Oocytes. Oocytes were surgically removed from mature *X. laevis* frogs and injected with 50 nl (5–20 ng) of appropriate nAChR subunit in vitro-transcribed RNAs as described previously (Papke and Stokes, 2010).

Oocyte Recording and Data Analysis. Experiments were conducted using OpusXpress 6000A (Molecular Devices, Sunnyvale, CA) and analyzed as described previously in detail (Papke and Stokes, 2010) using pClamp10 (Molecular Devices) and Excel software (Microsoft Corp., Redmond, WA). For all experiments evaluating the effects of PAMs, baseline conditions were defined by two control applications of 60 μM acetylcholine (ACh) made before the experimental applications. Test compounds were either applied into the bath using the OpusXpress system for changing the running buffer or with the normal OpusXpress pipette delivery system. Data were subsequently normalized by calculating responses relative to the average peak current and net charge of the two initial control responses. A period of 120 s was used for the calculation of net charge in all experiments beginning at the start of the solution application.

Chemicals and Synthesis of PNU-120596. Solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO), Thermo Fisher Scientific (Waltham, MA), and TCI America (Portland, OR). PNU-120596 was synthesized according to the method described previously (Piotrowski et al., 2003) for similar couplings. Specifically, reaction of 5-chloro-2,4-dimethoxyphenyl isocyanate (2.5 mmol) with 1 Eq of 3-amino-5-methyl isoxazole in 50 ml of dry benzene was maintained at 65°C for 4 days. The reaction mixture was cooled down and concentrated to a solid in vacuo. The crude product was recrystallized three times from isopropanol, decolorized with activated carbon in isopropanol, and then filtered through a diatomaceous earth (Celite) pad. After concentration, a fluffy white solid was obtained in 39% yield, having a melting point (219.5–220.5°C) identical to a commercially available sample from Tocris (St. Louis, MO). The purity and composition of PNU-120596 was further confirmed with NMR spectroscopy. ^1H NMR (300 MHz, acetone- d_6) δ 2.38 (d, J = 0.88 Hz, 3 H) 3.89 (s, 3 H) 3.96 (s, 3 H) 6.41 (br. s., 1 H) 6.87 (s, 1 H) 8.31 (s, 1 H) 8.85 (br. s., 1 H) 9.13 (s, 1 H). The mass spectrum was obtained on an Agilent 6210 time-of-flight spectrometer operated in electrospray ionization mode: m/z 334.0551 [$\text{M}+\text{Na}$] $^+$ (calculated, 334.0565). electrospray ionization.

Cell Culture and Transient Transfection of BOSC 23 Cells. BOSC 23 cells, obtained from American Type Culture Collection (Manassas, VA), were cultured at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with

10% fetal bovine serum in the absence of antibiotics. Cells were discarded and fresh cells thawed once 25 passages were reached. One day before transfection, cells were plated onto 12-mm glass coverslips (Thermo Fisher Scientific) previously coated with poly-D-lysine (Sigma-Aldrich). Cells were transiently transfected using FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. Plasmids (1, 0.3, and 0.8 μ g) containing cDNA clones of human $\alpha 7$, human RIC-3, and DsRed, respectively, were added with 6.3 μ l of FuGENE 6 reagent to each 35-mm dish containing cells and coverslips. Experiments were performed 48 to 72 h after transfection.

Outside-Out Patch Clamp Electrophysiology. Single-channel currents were recorded in the outside-out patch configuration using an Axopatch 200A amplifier (Molecular Devices) at room temperature. Cells were bathed in an external solution containing 165 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 10 mM glucose, 5 mM HEPES, and 0.001 mM atropine, pH adjusted to 7.3 with NaOH. Patch pipettes (Sutter Instruments, Novato, CA) were pulled to a tip diameter of 1 to 2 μ m, fire-polished to 5 to 10 M Ω , coated with SigmaCote (Sigma-Aldrich), and filled with an internal solution containing 147 mM CsCl, 2 mM MgCl_2 , 1 mM CaCl_2 , 10 mM EGTA, 10 mM HEPES, and 2 mM Mg-ATP, pH adjusted to 7.3 with CsOH. Recordings were low-pass-filtered to 10 kHz with the built-in amplifier filter (four-pole Bessel) and digitized at 100 kHz with a DigiData 1440 (Molecular Devices) using Clampex 10 data acquisition software (Molecular Devices).

Rapid drug application to outside-out patches was performed with a Burleigh piezoelectric stepper (EXFO, Ontario, Canada) as described previously (Williams et al., 2011a). θ -shaped glass (Sutter Instruments) was pulled, scored, and then broken by hand to create a drug application pipette with a diameter of 120 μ m (septum thickness, \sim 10 μ m) that was mounted to the stepper. The voltage signal used to control the piezoelectric stepper was conditioned by an RC circuit ($\tau = 2$ ms) to reduce oscillations and avoid damage to the crystal.

Two solution reservoirs (60 ml of Monoject syringes; Sherwood Medical, St. Louis, MO) were connected to each channel of the theta glass application pipette via polyethylene tubing; the tubing and application pipette could be completely primed with solutions from either reservoir within 15 s. Flow rates from each reservoir and channel of the drug application pipette were \sim 11 cm/s. Solution exchange times were typically between 0.4–0.7 ms (10–90% rise times) and were routinely determined by movement of diluted external solution over an open recording pipette. To maintain undisturbed laminar flow from the application pipette and minimize solution mixing, external saline solution was continuously perfused through the recording chamber (Warner Instruments, Hamden, CT) at a rate of 4 ml/min, and the application pipette was positioned such that streams flowing from it would directly enter the aspiration port of the chamber. In addition, the tip of the application pipette was kept free of dirt and/or cell debris by periodic cleaning in a hydrochloric acid solution.

Patch-clamp recordings were analyzed with Clampfit 10 (Molecular Devices, Union City, CA) and QuB (University at Buffalo, Buffalo, NY) software. Sections of data traces with minimal simultaneous openings and flanked by \geq 100 ms of closed time were selected for analysis. After initial periods of multichannel openings, single-channel bursts potentiated by PNU-120596 occurred in very obvious groups of openings that were separated by long silent periods. This characteristic eliminated the ambiguity that is normally associated with defining a burst delimiter value (t_{crit}) used in burst analysis of single-channel recordings (Colquhoun and Sakmann, 1995). The t_{crit} value used to determine average burst duration intervals was 150 ms; large variations in this value had minimal impact on the outcome of burst analysis. To determine intraburst closure, subconductance, and open durations, a total of 40 protracted bursts were selected at random for careful idealization. Data traces were idealized within QuB using the segmental k-means method (Qin, 2004) and were idealized at a bandwidth of 10 kHz, but the sampling rate

was reduced from 100 to 30 kHz to improve the quality of idealization because segmental k-mean idealization is generally optimized at or near the Nyquist frequency (F. Sachs, personal communication). After the automated idealization, the fit was manually inspected event-by-event, and corrections were made as necessary to the idealization. A temporal resolution limit of $1.3 \times$ filter rise time was set at 40 μ s and imposed in all analyses. The probability of channel opening was determined by the equation

$$NP_{\text{open}} = \frac{1}{iD} \int_0^D I(t) dt$$

where I is the recorded current relative to baseline, t is time, i is the mean single-channel amplitude, D is the duration of the drug application, and N is the number of channels in the patch. Of course, N is always unknown and the P_{open} measurement is only as accurate as the estimate of N .

Results

ACh-Evoked Responses of $\alpha 7$ nAChR Expressed in *X. laevis* Oocytes. The agonist-evoked responses of $\alpha 7$ nAChR have unique features that distinguish them from the responses of other nAChR subtypes and are important to consider to provide a context for how an allosteric modulator may work to increase those responses. Typically, the ACh-evoked responses of heteromeric nAChR increase during the application of agonist at a rate consistent with the speed of solution exchange and subsequently decay after solution exchange is complete, as illustrated in Fig. 1A. In our oocyte recording system the time constant for solution exchange is 3 to 4 s, matching the rise and decay of $\alpha 4\beta 2$ -mediated currents, in this case associated with the low-sensitivity form of the receptor containing three $\alpha 4$ and two $\beta 2$ subunits (Zhou et al., 2003). It should be noted that the apparent plateau of the $\alpha 4\beta 2$ -mediated currents during the agonist application does not indicate that these receptors do not desensitize but rather that the processes of activation and desensitization are both more rapid than the solution exchange and are therefore more or less at equilibrium during the plateau phase of the response (Papke, 2010). Because of the stability of these equilibrated responses during the agonist applications, the concentration-response relations for peak currents and net charge are essentially identical.

In contrast to responses of heteromeric nAChR, the responses of $\alpha 7$ nAChR evoked by the application of high concentrations of agonist (Fig. 1B) reach a peak before solution exchange is complete (Papke and Thinschmidt, 1998), so that large peak currents are indicative of synchronized activation but not increased receptor activation over the duration of the agonist application. This is reflected in the disparity between the apparent concentration-response relationships for $\alpha 7$ peak currents and net charge. It is therefore fundamentally a fallacy to attribute large peak current responses to high concentrations of agonist, and by inference high levels of agonist binding site occupancy, because little or no current is recorded under the conditions when agonist concentration is very high.

Activation of $\alpha 7$ nAChR occurs when there is only fractional occupancy of the agonist binding sites (Williams et al., 2011a), and the decreased P_{open} at higher levels of agonist occupancy is consistent with a form of agonist concentration-dependent desensitization unique to $\alpha 7$. One of the effects of

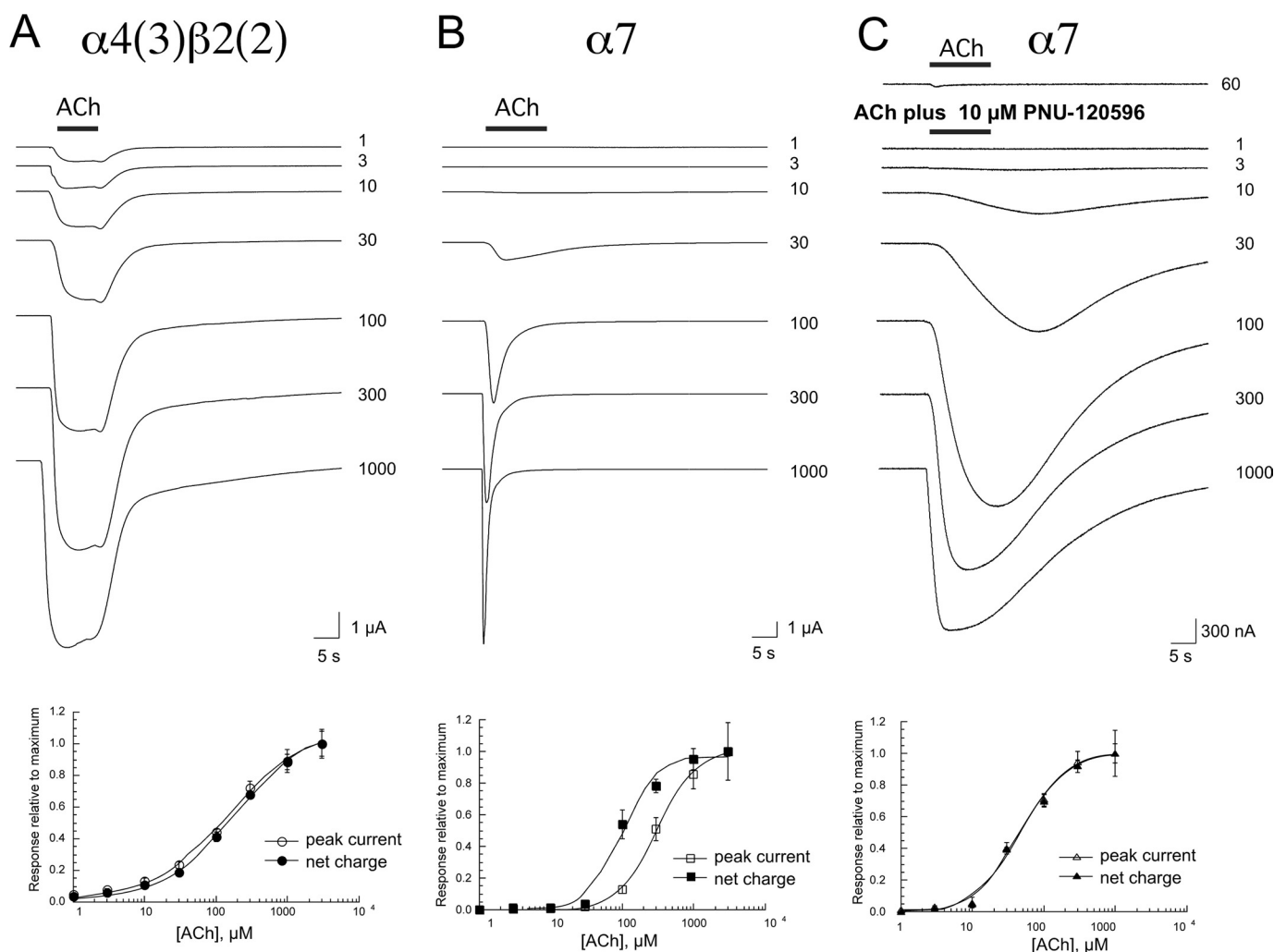


Fig. 1. The basic characterization of $\alpha 7$ macroscopic currents, comparison with currents from heteromeric $\alpha 4\beta 2$ nAChR, and the effects of PNU-120596. A, top, recordings from an *X. laevis* oocyte expressing human $\alpha 4\beta 2$ nAChR to the application of a range of ACh concentrations. Receptors with a fixed subunit stoichiometry of three $\alpha 4$ and two $\beta 2$ were obtained by coexpressing a concatamer of $\beta 2$ and $\alpha 4$ [$\beta 2$ -6- $\alpha 4$ (Zhou et al., 2003)] with monomeric $\alpha 4$. Bottom, average values for peak currents and net charge for the ACh-evoked responses of $\alpha 4\beta 2$ -expressing oocytes. The EC_{50} values for peak and net charge measurements were $157 \pm 28 \mu\text{M}$ and $184 \pm 25 \mu\text{M}$, respectively. Each point is the average of at least four cells \pm S.E.M. B, top, recordings from a *X. laevis* oocyte expressing human $\alpha 7$ nAChR to the application of a range of ACh concentrations. Bottom, average values for peak currents and net charge for the ACh-evoked responses of $\alpha 7$ -expressing oocytes. The EC_{50} values for peak and net charge measurements were 311 ± 15 and $98 \pm 12 \mu\text{M}$, respectively. Each point is the average of at least four cells \pm S.E.M. C, top, recordings from a *X. laevis* oocyte expressing human $\alpha 7$ nAChR to the application of a range of ACh concentrations obtained in the presence of 10 μM PNU-120596, compared with an initial control response 60 μM ACh alone from the same oocyte. Bottom, average values for peak currents and net charge of $\alpha 7$ -expressing oocytes for the ACh-evoked responses in 10 μM PNU-120596. The EC_{50} values for peak and net charge measurements were 48 ± 6 and $49 \pm 7 \mu\text{M}$, respectively. Each point is the average of at least four cells \pm S.E.M. After obtaining two initial control responses to the application of 60 μM ACh alone, the bath perfusion system was switched to a solution of Ringer's plus 10 μM PNU-120596. ACh applications were made with the pipette delivery system. ACh solutions were made up in the PNU-120596-containing bath solution. Net charge responses were calculated for a period of 120 s after the initiation of drug applications.

the type II $\alpha 7$ PAM PNU-120596 is to reverse or destabilize one or more forms of $\alpha 7$ receptor desensitization. As shown in Fig. 1C, the ACh-evoked responses of $\alpha 7$ nAChR recorded in the presence of PNU-120596 lacked the appearance of concentration-dependent desensitization. Consistent with the reversal of a form of desensitization that is unique to $\alpha 7$, in the presence of PNU-120596, the ACh concentration-response relationships for peak current and net charge were essentially identical, as is the case with heteromeric nAChR.

ACh-Evoked Responses of $\alpha 7$ nAChR Expressed in BOSC 23 Cells in the Absence and Presence of PNU-120596. A typical response of an outside-out patch expressing $\alpha 7$ to rapid application (≤ 0.7 ms) of 60 μM ACh is shown in Fig. 2A. Channel openings occur with highest frequency

immediately after application of ACh and then quickly disappear despite continued application of agonist. Individual $\alpha 7$ channel openings are extraordinarily brief and appear primarily as isolated events rather than bursts of openings. At a bandwidth of 10 kHz, the average duration of the apparent $\alpha 7$ single-channel openings evoked by 60 μM ACh is $113 \pm 7 \mu\text{s}$ ($n = 187$ openings, 13 patches). Considering that this short duration lies just above temporal resolution limits, many openings probably occurred that were too short to be reliably detected, and the average $\alpha 7$ open duration is overestimated. Figure 2B displays a histogram of the apparent channel openings fit by a single exponential function. Assuming the distribution of the missed openings follows the fit exponential function to the ordinate, the corrected average

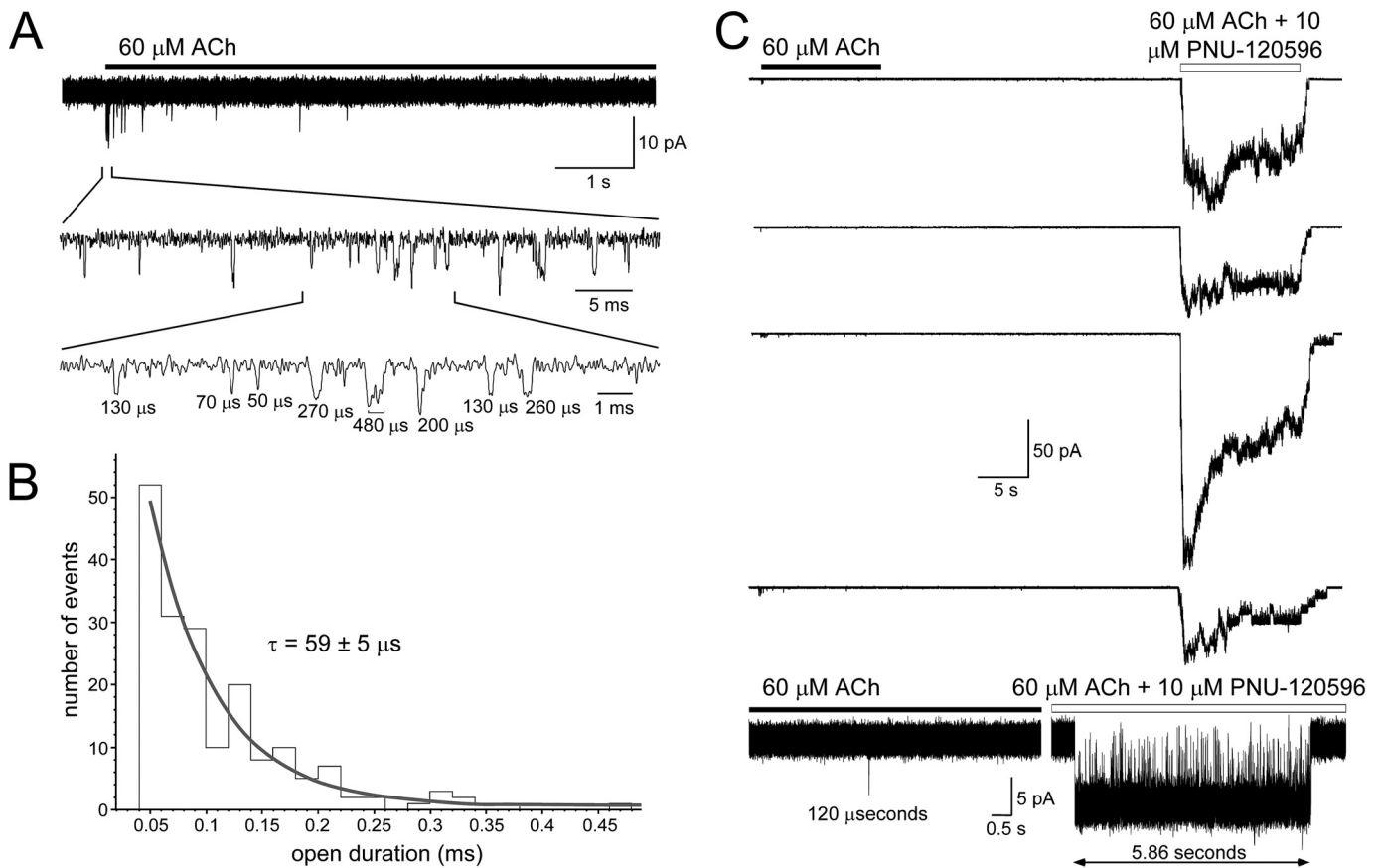


Fig. 2. The low intrinsic P_{open} of $\alpha 7$ is enhanced by PNU-120596. Data were obtained from outside-out patches pulled from BOSC 23 cells transiently expressing human $\alpha 7$ and RIC-3. **A**, a typical response evoked by rapid (≤ 0.7 -ms) application of 60 μM ACh showing the short-lived openings that are characteristic of $\alpha 7$. Notice that the frequency of channel openings quickly diminishes after exposure of the patch to ACh. **B**, a histogram of the apparent single-channel $\alpha 7$ open durations evoked by 60 μM ACh ($n = 187$ open events from 13 patches; 10 kHz bandwidth). Putative openings that could not be clearly distinguished from brief, random noise spikes were excluded from the histogram. The histogram is described by a single exponential function, which predicts that $\alpha 7$ single-channel openings are, on average, approximately 60 μs in duration, assuming that the distribution of openings too short to be detected ($< 40 \mu\text{s}$) reasonably follows the fit exponential function. **C**, responses produced by 60 μM ACh and relative increases produced with coapplication of with 60 μM ACh and 10 μM PNU-120596. Drug applications and interstimulus intervals were 12 and 30 s in duration, respectively.

$\alpha 7$ single-channel open duration is $59 \pm 5 \mu\text{s}$. At a holding potential of -70 mV , the average single-channel amplitude of the apparent $\alpha 7$ openings was $-6.22 \pm 0.08 \text{ pA}$. Given that individual $\alpha 7$ openings were so short in duration, this value may be an underestimate of the true single-channel amplitude. A scatterplot of channel amplitude versus open duration showed no clear overall relationship. However, all openings longer than 300 μs in duration had single-channel amplitudes between -6.8 and -7.6 pA (not shown).

The type II PAM PNU-120596 had a profound impact on responses evoked by 60 μM ACh from outside-out patches (Fig. 2C). In the absence of PNU-120596, responses evoked by 12-s applications of 60 μM ACh were limited due to the low P_{open} intrinsic to $\alpha 7$ and may lead one to believe the patch contained few channels. A follow-up application of 60 μM ACh with 10 μM PNU-120596 to the same patch revealed that in reality the patch contained a multitude of channels, demonstrating the massive potential for enhancement of $\alpha 7$ responses by PNU-120596. The net charge response evoked by coapplication of 60 μM ACh and 10 μM PNU-120596 was on average $1.35 \pm 0.42 \times 10^{-5}$ -fold larger than the net charge response evoked by 60 μM ACh alone ($n = 4$).

The peak current of the response evoked by ACh and PNU-120596 coapplication, divided by the unitary current

amplitude of PNU-120596-potentiated currents, provides a lower-limit estimate of the number of channels in a patch (N). The number of channels in each patch is an unknown but is a parameter of interest because it is required to calculate P_{open} . The ACh and PNU-120596 concentration combination that evokes maximal peak responses is important to know to obtain a lower-limit estimate of N that is as accurate as possible. We performed several experiments with *X. laevis* oocytes expressing $\alpha 7$ testing various concentration combinations of ACh and PNU-120596 and found that a range of ACh (10–100 μM) concentrations with 10 μM PNU-120596 provided comparable maximal peak currents (data not shown). We calculated, based on the detected $\alpha 7$ openings in the absence of PNU-120596 and the lower-limit of N provided by peak currents in response to 60 μM ACh and 10 μM PNU-120596 coapplication, an upper-limit estimate of $\alpha 7$ P_{open} in response to a 12-s application of 60 μM ACh of $7.4 \pm 3.0 \times 10^{-6}$ ($n = 4$). It is important to understand that this estimate is strictly an upper-limit estimate because N is unknown and potentially greatly underestimated. In addition, it is equally important to appreciate that this estimate includes the nonstationary portion of the 60 μM ACh-evoked responses, immediately after application of ACh, when channel activation occurs with highest probability, and that under

true steady-state conditions, the upper-limit $\alpha 7 P_{\text{open}}$ will be much less.

The Potentiation of $\alpha 7$ -Mediated Currents in *X. laevis* Oocytes by PNU-120596 Does Not Reflect the Functional Conversion of Previously Inactive Receptors. Aside from the unitary conductance and driving force, which we may assume are constants in all voltage-clamp experiments, macroscopic current responses such as those recorded in the *X. laevis* oocyte expression system depend on two factors that will vary from cell to cell and experiment to experiment: the total number of activatable receptors and the P_{open} of each single receptor. In theory, a PAM may operate on any of these factors to increase the magnitude of agonist-evoked responses. Because it has been reported that in some neuronal cell systems the majority of surface $\alpha 7$ receptors (McNerney et al., 2000) are not in fact readily activatable, we wished to determine whether PNU-120596 operated on the same population of receptors that were activatable in the absence of the PAM, or alternatively, increase the number of

receptors available for activation. To test this, we coapplied ACh with the slowly reversible use-dependent inhibitor bis-(2,2,6,6-tetramethyl-4-piperidiny)l sebacate (BTMPS; Papke et al., 1993) and measured the recovery of ACh-evoked responses 8 min later (Fig. 3A). At that time, there was still approximately 60% inhibition relative to the initial ACh-evoked responses. We then measured the responses stimulated by coapplications of 60 μM ACh and 10 μM PNU-120596 with or without prior BTMPS-induced inhibition. Responses were normalized to initial ACh controls, and we determined that the relative inhibition of the PNU-120596-potentiated responses was not different from the relative inhibition of the nonpotentiated responses (Fig. 3B), indicating that it is unlikely that PNU-120596 potentiation involves increasing the number of activatable receptors.

Convergence and Competition of Type I and II PAM Activity and PAM Effects on "Gain-of-Function" Mutants. Studies of chimeric receptors and receptors with site-directed mutations have implicated points of contact between

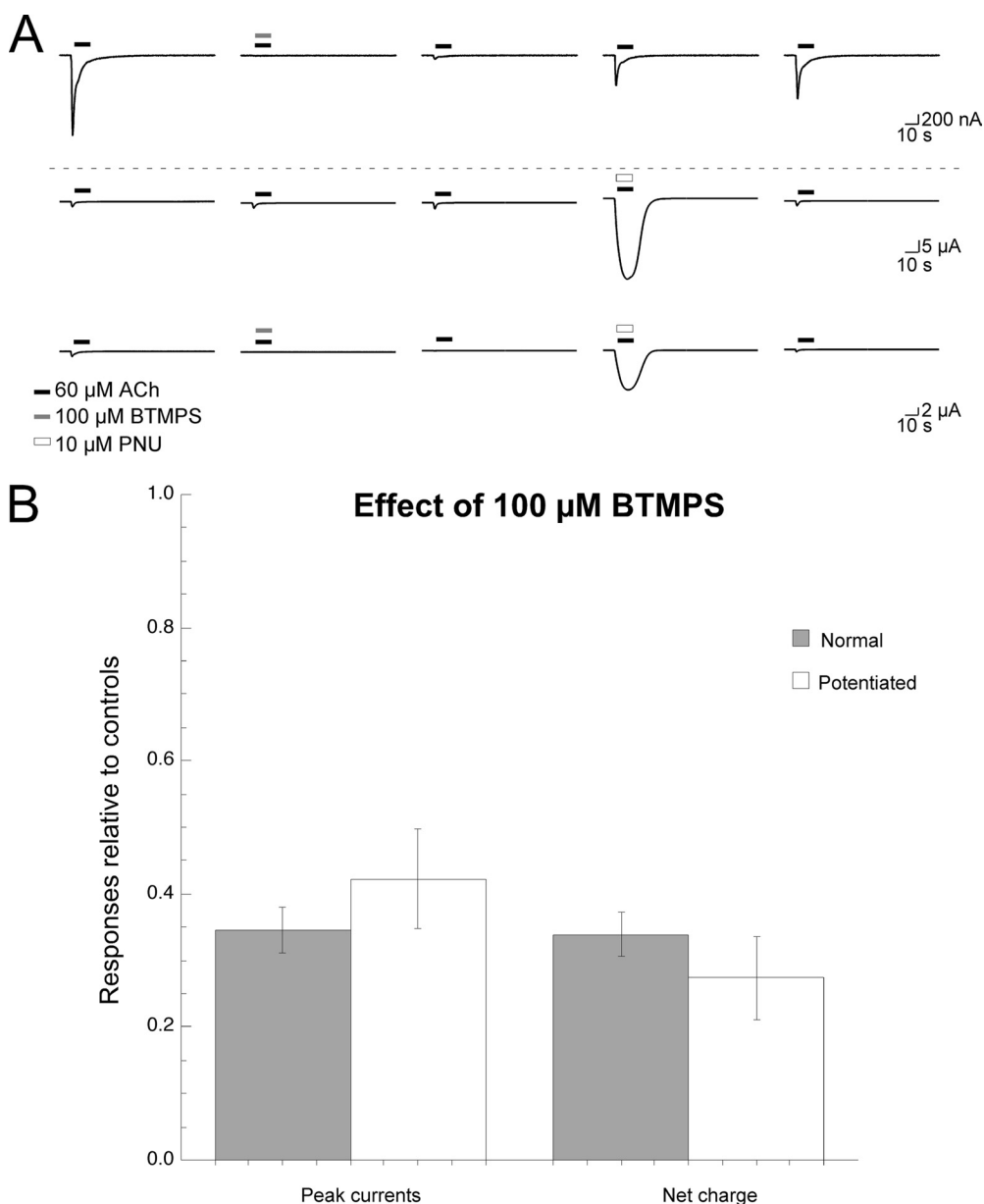


Fig. 3. PNU-120596 operates on the same population of receptors that are activatable in the absence of the PAM. To determine whether PNU-120596 increases the number of activatable receptors, we determined the effects of the slowly reversible use-dependent inhibitor BTMPS (Papke et al., 1993) with or without the potentiating effects of PNU-120596. A, sample traces illustrating the slow recovery of responses evoked by 60 μM ACh after a single coapplication of ACh with 100 μM BTMPS. Responses in the upper trace were obtained without PNU-120596. The lower two traces illustrate the effect of a single coapplication of PNU-120596 with or without a preceding coapplication of ACh and BTMPS. B, average data for the effect of BTMPS on the responses of at least four oocytes (\pm S.E.M.) obtained 8 min after a coapplication of ACh and the antagonist. Data obtained without PNU-120596 were normalized to the initial ACh-evoked responses. Data obtained from cells treated with both BTMPS and PNU-120596 went through a two-step normalization. Each cell's responses were first calculated relative to the initial ACh controls from the same cells. The responses to ACh plus PNU-120596 in the BTMPS-treated cells were then expressed relative to the average potentiation obtained in the cells treated with PNU-120596 without prior treatment with BTMPS.

extracellular and transmembrane domains as being important for mediating the potentiating effects of both type I and II PAMs, although it has been proposed that the actual binding site for PNU-120596 is in a water-filled space among the transmembrane α -helices (Young et al., 2008). As shown in Fig. 4A, coapplication of the prototypical type I PAM 5HI with PNU-120596 significantly reduced potentiation compared with the effects of PNU-120596 alone ($p < 0.05$). Such competition between type I and II PAMs might occur at either of two levels: through competition at a common bind-

ing site or through mechanistic interference at sites required to obtain the effects of either class of compound.

Multiple types of $\alpha 7$ "gain-of-function" mutations have been reported, most of which are associated with sequence changes in the second transmembrane domain (TM2), which is believed to line the ion channel. To varying degrees, the effects of these mutations mimic some of the effects of PAMs. We wished to determine the degree to which PAM activity relied on wild-type sequence at these sites and whether the activity of both type I and type II PAMs were perturbed in the

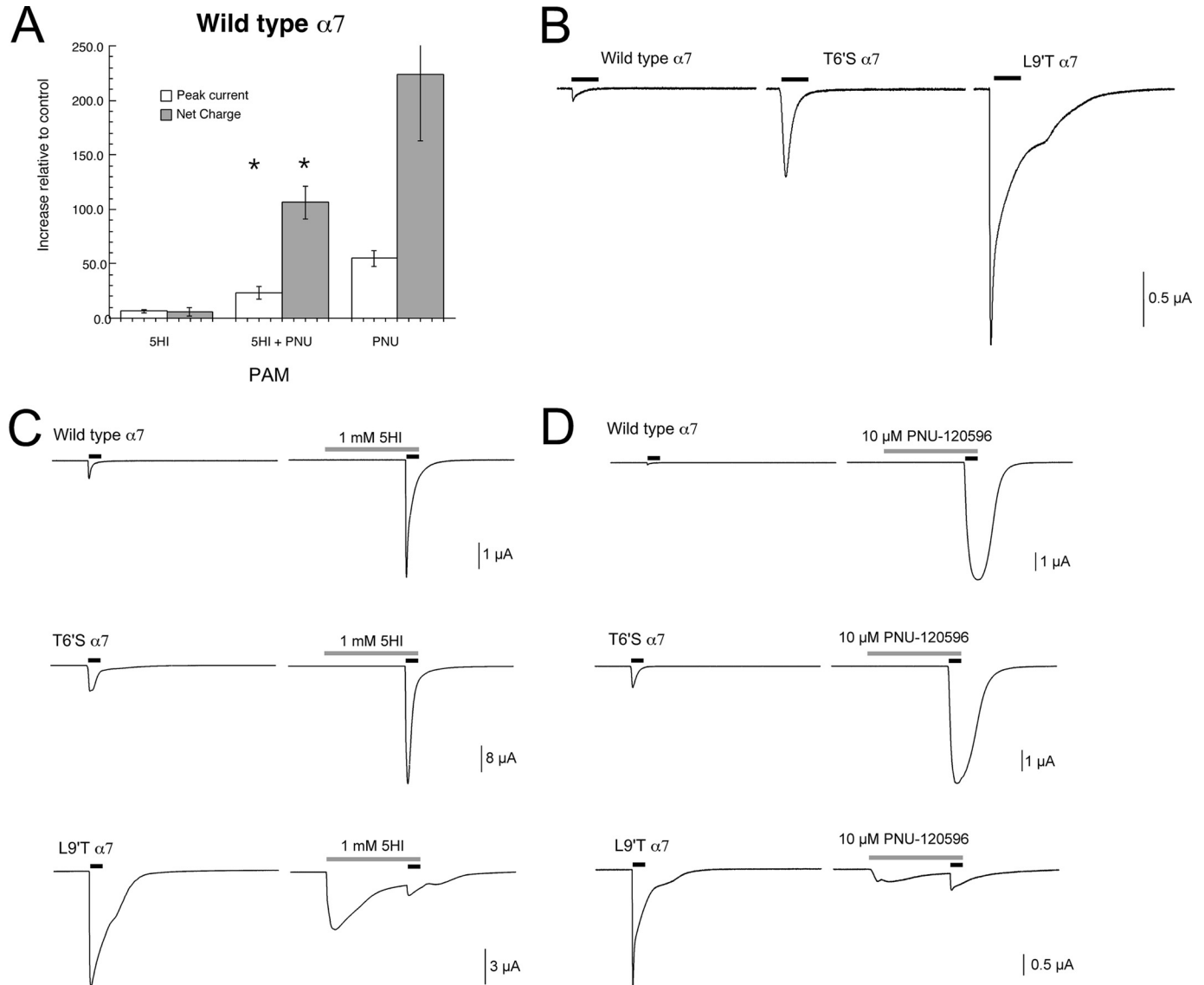


Fig. 4. Convergence and competition of types I and II PAM activity and PAM effects on "gain-of-function" mutants. **A**, competitive effects of the prototypical type I PAM 5HI on the potentiation of 60 μ M ACh-evoked responses of oocytes expressing human $\alpha 7$ nAChR. After obtaining control responses to 60 μ M ACh, responses were obtained to coapplications of ACh plus 1 mM 5HI, 10 μ M PNU-120596, or the two PAMs in combination. Data are the averages of at least four oocytes (\pm S.E.M.) for each condition. The coapplication of 5HI with PNU-120596 produced significantly less ($p < 0.05$) potentiation than that produced with PNU-120596 alone. **B**, representative traces illustrating the effects of two gain-of-function mutations in the second transmembrane domain of $\alpha 7$ nAChR. The effects of the T6'S mutation (Placzek et al., 2005) are qualitatively similar to those of type I PAMs, whereas the effects of the well studied L9'T mutation are greater (Bertrand et al., 1997), especially in regard to the characteristic rapid desensitization of $\alpha 7$, and have been compared with the effects of a type II PAM (Gronlien et al., 2007). **C**, the effects of the T6'S and L9'T mutations on the activity of the type I PAM 5HI. Although, as expected, the ACh control responses of $\alpha 7$ T6'S receptors were larger than the responses of wild-type $\alpha 7$ receptors, the effects of 5HI pre- and coapplications were relatively similar to effects on wild-type receptors. In contrast, $\alpha 7$ L9'T responded to the preapplication of 5HI alone, and responses during the coapplication of 5HI and ACh were smaller than to ACh alone. **D**, the effects of the T6'S and L9'T mutations on the activity of the type II PAM PNU-120596. Although, as expected, the ACh control responses of $\alpha 7$ T6'S receptors were larger than the responses of wild-type $\alpha 7$ receptors, the effects of PNU-120596 pre- and coapplications were relatively similar to effects on wild-type receptors. In contrast, $\alpha 7$ L9'T responded to the preapplication of PNU-120596 alone, and responses during the coapplication of PNU-120596 and ACh were smaller than to ACh alone. In all traces (B–D), the black bars represent 20-s applications of 60 μ M ACh.

same manner and to the same degree by specific mutations. The substitution of a serine for threonine at the sixth residue in the $\alpha 7$ TM2 ($\alpha 7T6'S$; Placzek et al., 2005) produces a modest gain of function with features resembling those produced by a type I PAM. The peak macroscopic currents of $\alpha 7T6'S$ mutants are large compared with wild-type $\alpha 7$, and single-channel open times are prolonged, sometimes occurring in short bursts (Placzek et al., 2005). However, as is the case with wild-type receptors modified by a type I PAM, the currents of $\alpha 7T6'S$ mutants decay to baseline during the application of a high concentration of agonist (Fig. 4B). These effects of the T6'S mutation are relatively subtle compared with the well studied mutation at the 9' position in TM2, L9'T (Bertrand et al., 1997). The effects of this mutation are so extensive that it might arguably be called a "perversion-of-function" as much as a "gain-of-function" mutation, because, unlike the T6'S mutants which preserve the pharmacological distinction between agonists and antago-

nists, $\alpha 7L9'T$ mutants may be activated by agents that are antagonists of wild-type receptors. The $\alpha 7L9'T$ mutants generate currents that are very large (Fig. 4B), and it has been proposed that the mutation destabilizes a desensitized state or converts it into a conducting conformation, an effect that has also been proposed for the potentiation of wild-type $\alpha 7$ by PNU-120596.

As shown in Fig. 4C, the effects of the type I PAM 5HI on $\alpha 7T6'S$ receptors were similar to the effects on wild-type $\alpha 7$, although proportionately somewhat smaller due to higher initial control responses. In contrast, 5HI behaved more like an agonist than a PAM on $\alpha 7L9'T$ receptors because activation occurred in response to the preapplication of 5HI alone and was not much increased when ACh was coapplied. In fact, the responses of the $\alpha 7L9'T$ receptors to ACh in the presence of 5HI were less than responses to ACh alone. Likewise, the effects of PNU-120596 on $\alpha 7T6'S$ receptors were similar to the effects on wild-type $\alpha 7$, albeit somewhat

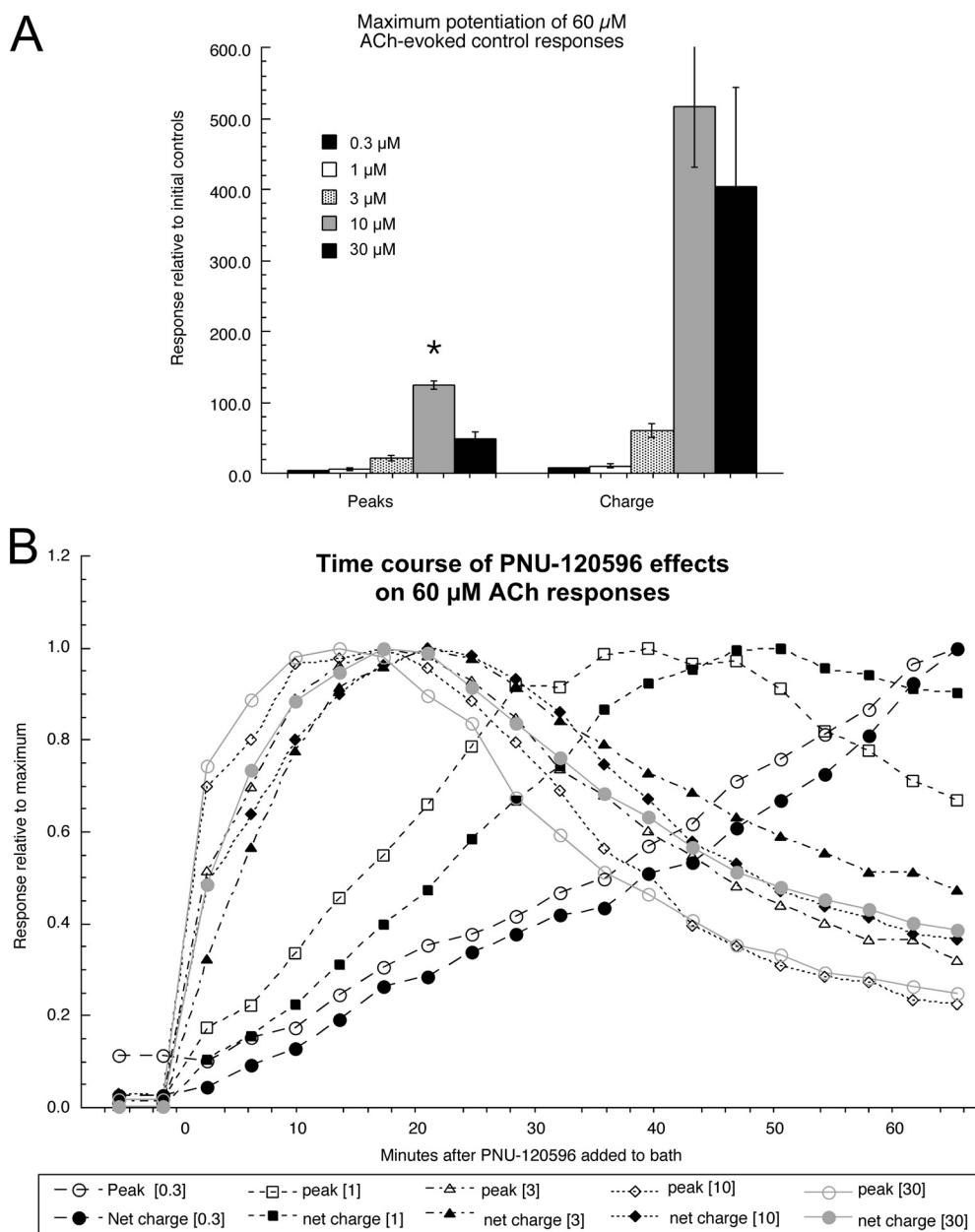


Fig. 5. Factors defining and limiting the potentiating effects of PNU-120596 on $\alpha 7$ nAChR expressed in *X. laevis* oocytes. **A**, maximum potentiation of 60 μ M ACh-evoked responses obtained with different concentrations of bath-applied PNU-120596 applied repeatedly at 4-min intervals for more than 60 min. **B**, the time course of the potentiation of 60 μ M ACh-evoked responses by varying concentrations of bath-applied PNU-120596. Note that although each point represents the average of at least four oocytes, error bars have been omitted for clear presentation of the kinetic differences. In the presence of 0.3 μ M PNU-120596, potentiation was significantly ($p < 0.01$) larger at 64 min than at 12 min. In the presence of 1.0 μ M PNU-120596, potentiation was greater at 40 min than at 12 min ($p < 0.05$), but not significantly greater at 64 min than at 12 min. For all concentrations of PNU-120596 ≥ 3 μ M, the potentiation at 64 min was significantly less than that at 12 min ($p < 0.05$).

smaller compared with initial controls, whereas the $\alpha 7$ L9'T mutants showed a response to the application of the PAM in the absence of agonist, and smaller responses to ACh plus PNU-120596 than to ACh alone (Fig. 4D).

These data suggest that the effects of the T6'S mutation are not additive, synergistic, or antagonistic to the effects of the PAMs tested, whereas the $\alpha 7$ L9'T mutant is a poor model for the mechanism of PNU-120596 potentiation. Although both PNU-120596 and the mutation appear to disrupt a form of desensitization that is unique to $\alpha 7$, the effects of PNU-120596 still have a strict requirement for some agonist binding.

Factors Limiting the Potentiating Effects of PNU-120596 on $\alpha 7$ -Mediated Currents. We investigated the time and concentration dependence of PNU-120596 potentiation of wild-type human $\alpha 7$ nAChR responses by making repeated applications of 60 μ M ACh in the presence of varying bath concentrations of PNU-120596 over the course of 1 h. Maximal potentiation of peak current amplitude was achieved with 10 μ M PNU-120596, although the maximal potentiation of net charge was similar in the presence of both 10 and 30 μ M (Fig. 5A). Interesting concentration-dependent effects were seen on the onset and decline of potentiation (Fig. 5B). In the presence of 0.3 μ M PNU-120596 potentiation increased throughout the experiment, with potentiation significantly ($p < 0.01$) larger at 64 min than at 12 min. In the presence of 1 μ M PNU-120596, potentiation was greater at 40 min than at 12 min ($p < 0.05$), but not significantly greater at 64 min than at 12 min. For all concentrations of PNU-120596 ≥ 3 μ M, the potentiation reached a maximum level within approximately 10 to 20 min and then decayed, with potentiation significantly less at 64 min than that at 12 min ($p < 0.05$). These data support the hypothesis that, although some of the mechanism of PNU-120596 may involve the relief or reversal of a sensitive form of desensitization (D_s), strong or protracted stimulation in the presence of PNU-120596 promotes the conversion of the receptor into an alternative PNU-120596-insensitive desensitized state (D_i).

To evaluate the ACh concentration dependence of responses potentiated by PNU-120596, 1 μ M and 100 μ M ACh were applied to an outside-out patch expressing $\alpha 7$ with the PNU-120596 concentration fixed at 10 μ M (Fig. 6). Neither the peak current nor the net charge were different in these experiments between responses evoked by 1 μ M ACh or 100 μ M ACh in the presence of 10 μ M PNU-120596. However, the onset and decay of the potentiation were different. Responses evoked by 1 μ M ACh in the presence of 10 μ M PNU-120596 had significantly ($p < 0.05$) slower 10-to-90% rise times than responses evoked by 100 μ M ACh and 10 μ M PNU-120596 (12.3 ± 5.1 versus 1.4 ± 0.5 s; $n = 4$). Responses evoked by 100 μ M ACh tended to peak relatively rapidly and then decay despite the continued application of ACh and PNU-120596, whereas responses evoked by 1 μ M ACh generally showed little to no decay on the time scale of these recordings. The ratio of the net charge during the last quarter of the response relative to the first quarter of the response was 1.35 ± 0.28 with 1 μ M ACh, whereas the ratio was 0.39 ± 0.08 in the 100 μ M ACh condition. The average 90-to-10% decay time of 100 μ M ACh and 10 μ M PNU-120596 responses was 26.14 ± 3.52 s ($n = 4$).

To test the hypothesis that the induction of D_i is dependent on receptor activation, we treated oocytes with one of two

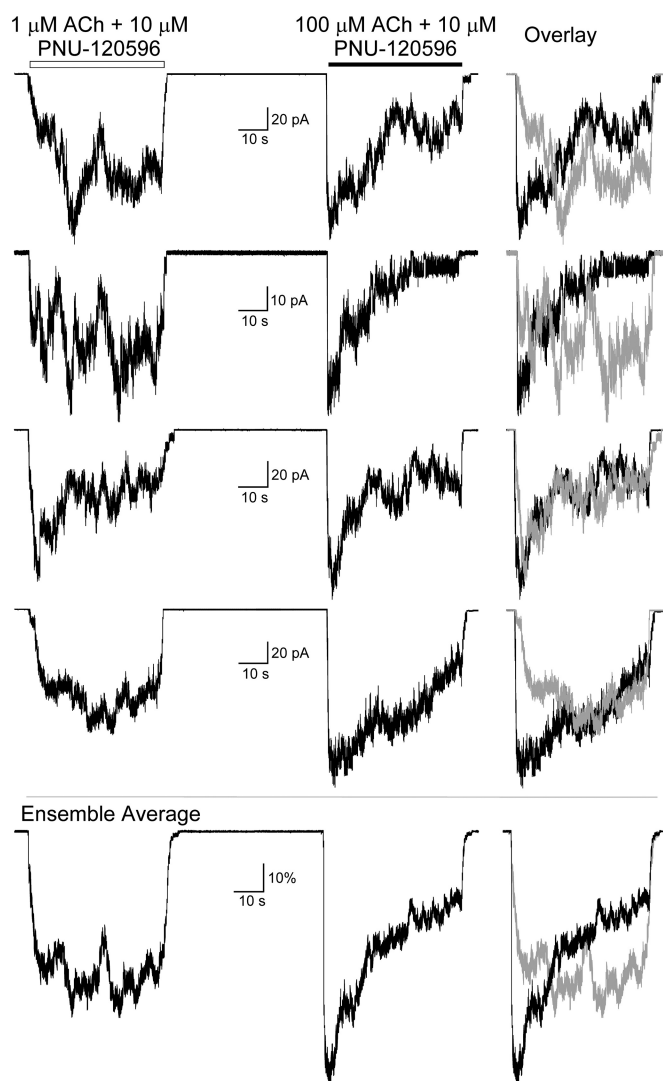
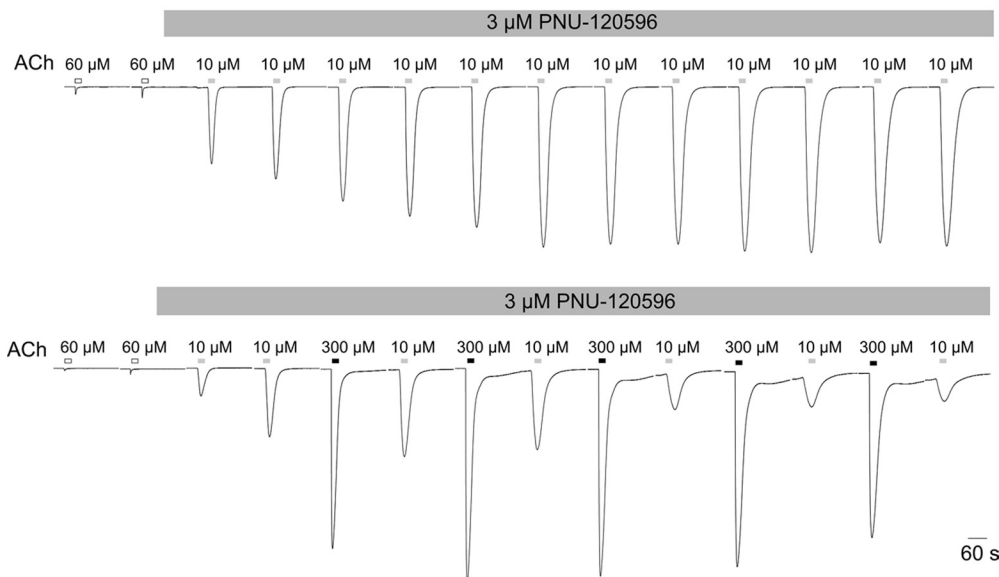


Fig. 6. Agonist concentration dependence on the onset and decline of potentiation by PNU-120596 in outside-out patches containing $\alpha 7$ receptors. Comparison of responses from the same patch potentiated by 10 μ M PNU-120596 and evoked by 1 or 100 μ M ACh. Drug applications and interstimulus intervals were 45 and 55 s in duration, respectively. The ensemble average of all four current traces was created by first normalizing each data point of an individual trace to the peak current of that trace. Then, the average value of the normalized currents was calculated for each data point.

different stimulus protocols (Fig. 7A). In the continued presence of 3 μ M PNU-120596, oocytes were either repeatedly stimulated with 10 μ M ACh or with 10 μ M ACh applications in alternation with stronger stimulation by 300 μ M ACh. As shown in Fig. 7B, the 10 μ M ACh-evoked responses of cells stimulated alternately with 300 μ M ACh reached a level of maximal potentiation relatively quickly and then showed a decline, consistent with the induction of D_i . In contrast, the 10 μ M ACh-evoked responses of oocytes that did not receive alternating application of 300 μ M ACh were slower to reach the same level of maximal potentiation, but also no decline in potentiation occurred over the course of an hour. Together these results suggest that both agonist and PNU-120596 concentrations affect the time course of potentiated responses, with faster onset of potentiation and subsequent decay occurring at high concentrations, and low concentra-

A

Activity dependence of potentiation



B

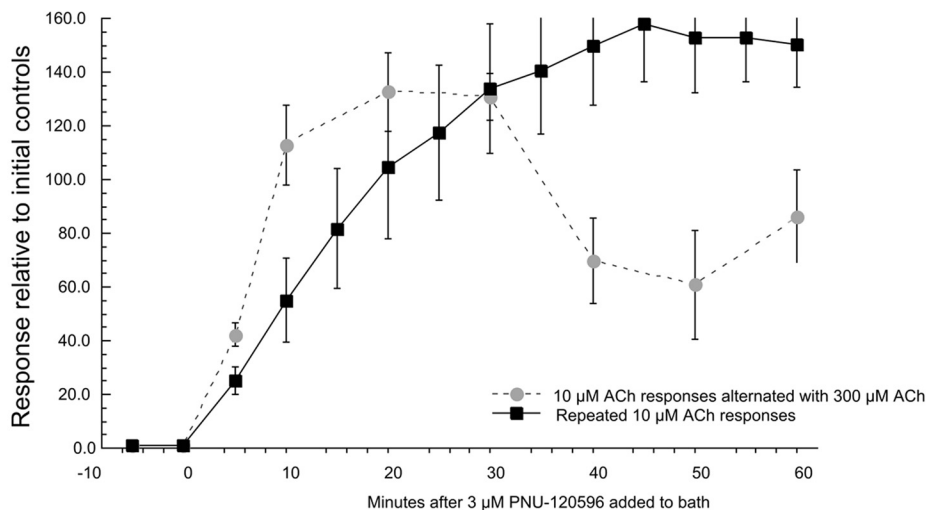


Fig. 7. Activity dependence of potentiation onset and decline in *X. laevis* oocytes. A, representative raw data showing the repeated stimulation of $\alpha 7$ -expressing oocytes in the presence of 3 μ M PNU-120596. Cells were stimulated repeatedly with either 10 μ M ACh (upper trace) or 10 μ M ACh alternating with applications of 300 μ M ACh (lower trace). B, average data (\pm S.E.M.) for 10 μ M ACh-evoked responses obtained with the two protocols illustrated in A. Note that maximal potentiation was achieved more rapidly when there were alternating applications of the high concentration of ACh; however, potentiation subsequently declined.

tions producing relatively slow rising but longer-lasting currents.

Although $\alpha 7$ nAChRs desensitize rapidly in response to high concentrations of agonist, the desensitization is also rapidly reversible once agonist has dissociated from the receptor (Papke et al., 2000). We tested whether the PNU-120596-insensitive form of desensitization that developed with strong stimulation in the presence of the PAM was also readily reversible. As shown in Fig. 8A, if repeated coapplications of 60 μ M ACh and 10 μ M PNU-120596 to oocytes were stopped during the period of time when insensitive desensitization was developing and later reinitiated, the receptors became resensitized to the effects of the PAM. Likewise, recovery from D_i induced by 300 μ M ACh and 10 μ M PNU-120596 occurred in outside-out patches in experiments performed with a much shorter timescale (Fig. 8B).

Single-Channel Bursts of $\alpha 7$ nAChR Promoted by PNU-120596. Because the potentiation of responses by PNU-120596 is so profound, we initially reasoned that the probability of observing single-channel currents potentiated

by PNU-120596 would be low. However, the decay of current was so pronounced with prolonged long applications of 300 μ M ACh and 10 μ M PNU-120596 that single-channel bursts potentiated by PNU-120596 were revealed once the majority of channels entered the D_i state(s) (Fig. 9A). From three patches with sections of continuous data >60 s lacking simultaneous channel openings, the steady-state absolute P_{open} (NP_{open}) in 300 μ M ACh and 10 μ M PNU-120596 was 0.40 ± 0.04 . Given the length of the single-channel bursts and the lack of simultaneous openings in these traces, it seems that the number of activatable channels at any time is greatly limited by D_i . It is unknown whether the openings in these traces all arise from one resilient channel that escaped and remained resistant to D_i , or whether the openings arose from multiple channels as they temporarily escaped D_i .

There seemed to be two qualitatively distinct groups of single-channel openings in the presence of PNU-120596 (Fig. 9A). One class of PNU-120596-potentiated opening appeared as relatively brief events that primarily occurred in isolation, and the other, more common, type of PNU-120596-potenti-

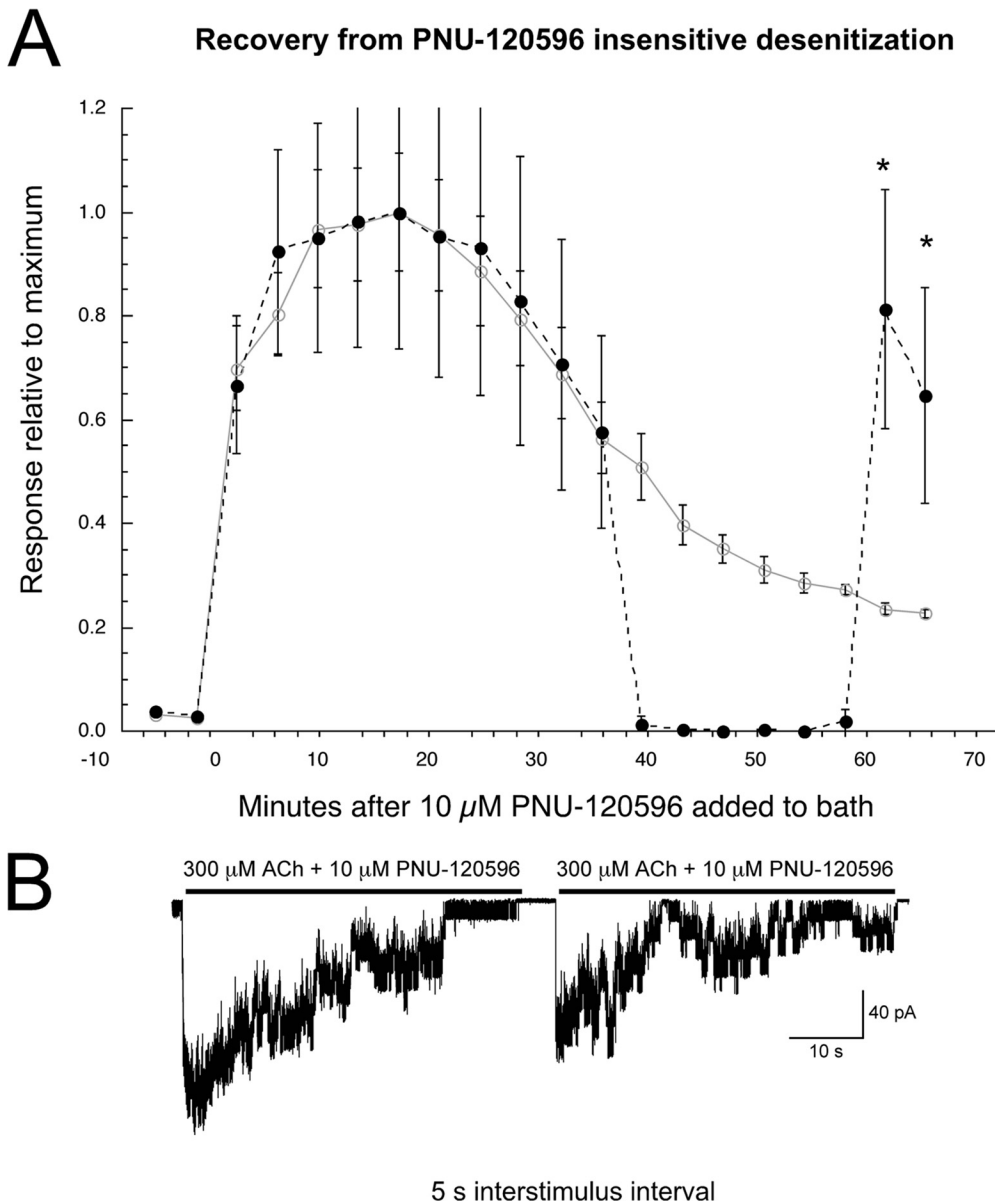


Fig. 8. Recovery from PNU-120596-insensitive desensitization (D_i). **A**, *X. laevis* oocytes expressing human $\alpha 7$ were stimulated repeatedly with 60 μ M ACh in the presence of 10 μ M PNU-120596. The amount of potentiation rapidly reached a maximum and then began to decline. To determine whether the decline in ACh-evoked responses represented a reversible form of PNU-120596 desensitization, some cells (solid points) received six applications of PNU-120596 solution alone rather than repeated coapplications of ACh plus PNU-120596. Subsequent application of ACh plus PNU-120596 to the cells that had received a respite from the repeated ACh stimulations gave responses that returned to a level that was not different from the previously maximal level of potentiation, and significantly greater than ($p < 0.01$) that of the cell receiving continuous stimulation. **B**, responses from outside-out patches expressing $\alpha 7$ to coapplication of 300 μ M ACh and 10 μ M PNU-120596 show a rapid rise and subsequent decay despite the continued presence of ACh and PNU-120596, indicative of stabilized D_i states. Recovery from D_i induced by coapplication of 300 μ M ACh and 10 μ M PNU-120596 occurs rapidly. Identical 45-s drug applications were separated by 5-s interstimulus intervals.

ated openings seemed to occur as long bursts of openings separated by very short closures to either fully closed or subconductance states. Most of the brief type of openings observed in the presence of PNU-120596 were <1 ms in duration, occasionally lasting approximately 8 ms (Table 1). A major component of the isolated openings evoked in the presence of PNU-120596 was fit with a time constant of 120 ± 6 μ s, which is similar to the average apparent open duration in the absence of PNU-120596.

On average, the long bursts of openings potentiated by 10 μ M PNU-120596 persisted for 5.48 ± 0.40 s ($n = 217$, 26 patches). The majority of the apparent intraburst closures were approximately 100 to 200 μ s; however, occasionally intraburst closures longer than approximately 10 ms were observed. The subconductance events were approximately 400 μ s or less in duration and were interrupted by many fast full channel closures (Fig. 9A, inset; Table 1). There appeared to be two distinct classes of the long PNU-120596-potentiated bursts; the distinction was made on the basis of the duration of the intraburst openings (Fig. 9B; Table 1). Approximately

27% of the bursts analyzed exclusively contained intraburst openings of approximately 5 ms in duration (class I bursts), and 73% of the bursts exclusively contained intraburst openings of approximately 30 ms in duration (class II bursts). No clear relationship was apparent between burst length and average intraburst open durations (Fig. 9B) and the apparent closed and subconductance duration intervals were similar between the two classes of bursts (Table 1). The reason for the two types of bursts is unclear but may be related to occupancy of ACh and/or PNU-120596 at their respective binding sites during the burst of openings.

At a holding potential of -70 mV, the single-channel amplitude of openings potentiated by PNU-120596 was -7.76 ± 0.08 pA, and the subconductance level amplitude was -3.59 ± 0.02 pA. The current-voltage relationship of currents evoked in the presence of PNU-120596 showed strong inward rectification, a reversal potential of -6.93 ± 1.01 mV, and a single-channel chord conductance of 129 ± 3 pS through the linear section of the relationship ($n = 6$, data not shown). Given that PNU-120596 seems to have little, if any,

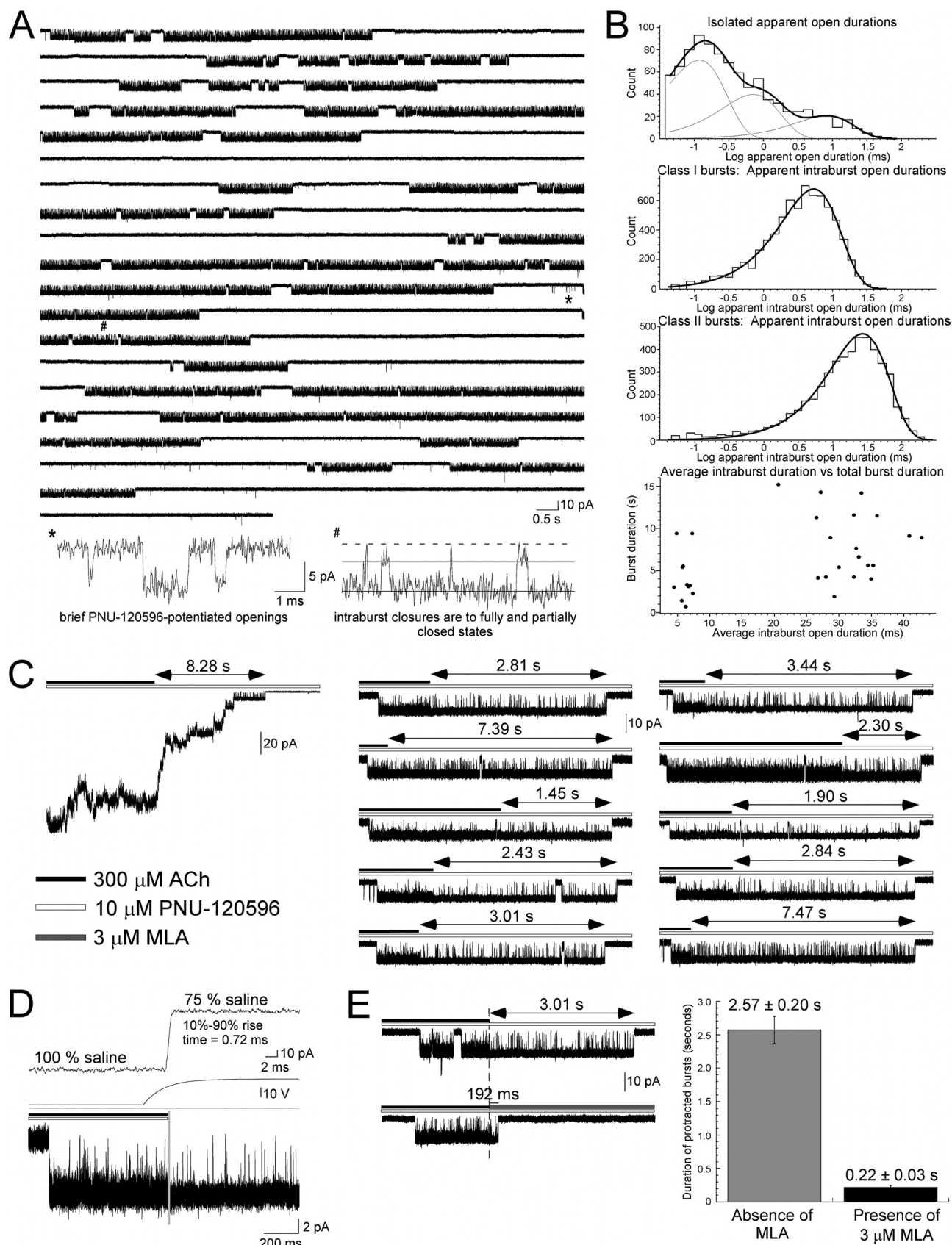


Fig. 9. Single-channel $\alpha 7$ bursts evoked by 300 μ M ACh and potentiated by 10 μ M PNU-120596. **A**, continuous data demonstrating the bursting characteristics and high steady-state P_{open} of single-channel openings potentiated by PNU-120596. **B**, fit histograms displaying apparent open durations from $\alpha 7$ channels activated by 300 μ M ACh and potentiated by 10 μ M PNU-120596. Shown are histograms compiled from currents potentiated by PNU-120596 that occur as brief events in isolation and in long groups of openings as bursts. Two classes of bursts are distinguished

TABLE 1

Fit time-constants from event duration histograms in the presence of 300 μ M ACh and 10 μ M PNU-120596Data obtained from outside-out patches pulled from BOSC 23 cells. *P* values indicate fraction of total events estimated from histogram fit. Values are presented as mean \pm S.E.M.

Type of Event	Isolated Openings		Class I Bursts		Class II Bursts	
	<i>P</i>	τ	<i>P</i>	τ	<i>P</i>	τ
		<i>ms</i>		<i>ms</i>		<i>ms</i>
Intraburst closures	N.A.		0.91 ± 0.06	0.07 ± 0.10	0.77 ± 0.14	0.16 ± 0.18
			0.07 ± 0.04	0.51 ± 0.82	0.21 ± 0.15	0.57 ± 0.61
			0.02 ± 0.03	9.16 ± 4.52	0.02 ± 0.04	13.2 ± 2.5
Intraburst subconductances	N.A.		0.80 ± 0.12	0.08 ± 0.09	0.82 ± 0.07	0.14 ± 0.09
			0.20 ± 0.04	0.40 ± 0.20	0.18 ± 0.09	0.37 ± 0.24
Openings or intraburst openings	0.54 ± 0.02	0.12 ± 0.06				
	0.30 ± 0.02	0.71 ± 0.11				
	0.16 ± 0.02	8.23 ± 0.14	1	5.35 ± 0.02	1	27.25 ± 0.02

N.A., not applicable.

effect on channel conductance (Hurst et al., 2005), the conductance measured in the presence of PNU-120596 is likely to be a reasonable estimate of single-channel $\alpha 7$ conductance in the absence of PNU-120596. This measurement is higher than the previously published values of 60 to 90 pS (McNerney et al., 2000; Mike et al., 2000), but the difference is probably related to the limited ability to accurately measure $\alpha 7$ open channel amplitude in the absence of PAM, because nonpotentiated $\alpha 7$ openings are so brief.

A phenomenon observed with currents from the outside-out patches that had been activated with ACh and potentiated by PNU-120596 was a postponed return to baseline after the removal of external ACh (Fig. 9C). For single-channel bursts activated in the presence of 300 μ M ACh and 10 μ M PNU-120596, currents persisted for 2.57 ± 0.2 s after the removal of external ACh ($n = 143$, 15 patches). There was no detectable change of intraburst open durations, and there was no apparent trend toward change in intraburst open duration over time after ACh removal. The protracted currents were not an artifact of slow or incomplete solution exchange, on the basis of three arguments. First, after data collection, each patch was blown off the tip of the pipette, and solution exchange profiles were determined by measuring changes in holding current upon moving diluted saline over the open pipette tip with the piezoelectric stepper (Fig. 9D). Data were analyzed only if the solution exchange profile measured in this way was clean and occurred rapidly, within ~ 700 μ s (10–90% rise time). Typical solution exchange times measured with this method were between 400 and 700 μ s. Second, there was a sharp reduction in current noise that was intimately associated with the removal of external ACh (Fig. 9D). This reduction in noise was probably due to relief of channel block by ACh, given that a relatively high concentration was used as the stimulating agonist (Colquhoun and Sakmann, 1985). This reduction in current noise was highly significant ($p < 3 \times 10^{-15}$), as measured by changes in the S.D. from the mean open current before and after removal of external ACh ($\sigma = 0.44 \pm 0.008$ versus 0.31 ± 0.003). Third,

replacement of external ACh with the competitive $\alpha 7$ antagonist methyllycaconitine (MLA) greatly attenuated the duration of the protracted currents (see below). The currents that persisted long after the removal of external ACh are consistent with the prolonged retention of ACh at the binding sites when PNU-120596 is applied. However, this observation could also be explained by maintenance of the channel in a reverberating conducting condition by PNU-120596 after dissociation of ACh on termination of external ACh application.

We attempted to test the hypothesis that PNU-120596 prolongs the retention of ACh to its binding site by removing external ACh and replacing it with 3 μ M MLA. We initially reasoned that if the affinity of the agonist-binding site for ACh was increased by PNU-120596, application of the high MLA concentration would have little effect on the duration of protracted currents, given that the channel was both occupied by ACh and in an activated condition on application of MLA. When ACh was replaced with MLA, the currents persisted for 0.22 ± 0.03 s ($n = 25$, three patches) after removal of external ACh, a significant ($p < 10^{-5}$) reduction of the protracted current duration (Fig. 9E).

It is noteworthy that the effect of MLA, which is normally considered a competitive antagonist, had the appearance of an inverse agonist in these experiments, given that channels were active when MLA was applied, and MLA shortened burst activity under conditions when no external ACh was available for competition. Before moving the patches of the solution containing 300 μ M ACh, we believe it is reasonable to assume that the five agonist binding sites of $\alpha 7$ were occupied by ACh at high probability. Furthermore, we would hypothesize that after the removal of external ACh, agonist molecules began to dissociate one-by-one until the last ACh molecule dissociated, perhaps resulting in termination of the burst. When external ACh was removed and individual ACh molecules began to dissociate, MLA may have had the opportunity to bind the receptor in the vacant binding sites and terminate the burst before all ACh molecules had dissociated. Our data suggest that binding of only one or two mol-

based on the average intraburst open duration, which is demonstrated below with a scatter plot of average intraburst duration versus total burst length. C, PNU-120596-potentiated channel openings persist for seconds after removal of external ACh. Left, an example of a protracted macroscopic current that persisted for approximately 8.3 s after external ACh removal. Right, single-channel bursts showing protracted currents of various durations after external ACh removal. D, the protracted currents were not an artifact of solution exchange. Above, solution exchanges typically occurred within 400 to 700 μ s, as measured by open-tip recordings performed after data collection from each patch. Below, a sharp reduction in current noise was associated with the removal of external ACh. The noise was probably due to channel block by agonist, which disappeared as external agonist was removed. E, application of the competitive antagonist MLA at 3 μ M after removal of external ACh significantly ($p < 10^{-5}$) shortened the duration of protracted currents. Both currents displayed in E were obtained from the same patch.

ecules of MLA may be sufficient to inactivate the entire receptor and that MLA may actively inhibit channel gating, perhaps by stabilizing nonconducting receptor conformations, rather than simply occupying the cavity where ACh binds to produce channel activation.

Multiple Phases of PNU-120596-Promoted Currents during Prolonged Coapplications with Agonists in *X. laevis* Oocytes. Because PAMs are in therapeutic development, it is important to characterize their activity under conditions when they are present for long periods of time at relatively fixed concentrations. Likewise, we do not know the temporal character of the ACh and/or choline presentation to $\alpha 7$ nAChR in the brain, which may well involve oscillations on the time scale of seconds or longer. Therefore, we investigated the time and concentration dependence of PNU-120596/agonist interactions on a protracted time scale to characterize both transient and potential steady-state effects.

Because of the induction of D_i as a function of the level of

receptor activation, it was expected that there would be variations in the time course and level of equilibrium potentiation, depending on the agonist and PNU-120596 concentrations. The multiple phases of $\alpha 7$ receptor activation produced by bath coapplication of 3 μ M PNU-120596 and varying concentrations of ACh are shown in Fig. 10A (all traces are scaled relative to the amplitude of the initial ACh controls). There were two phases of potentiated current in all of these evoked responses: a fast transient peak and a subsequent slow phase. The peaks of the transient currents occurred faster with higher concentrations of ACh (Fig. 10C). Although the timing of the transient peaks changed progressively through the range of ACh concentrations tested, the heights of the transient currents were greatest for the 30 μ M ACh and 3 μ M PNU-120596 coapplications (Fig. 10D).

The slow phases of the potentiated responses were also dependent on ACh concentration. For example, the slow phase of the response evoked by 300 μ M ACh and 3 μ M PNU-120596 shown in Fig. 10A reached a peak and began to

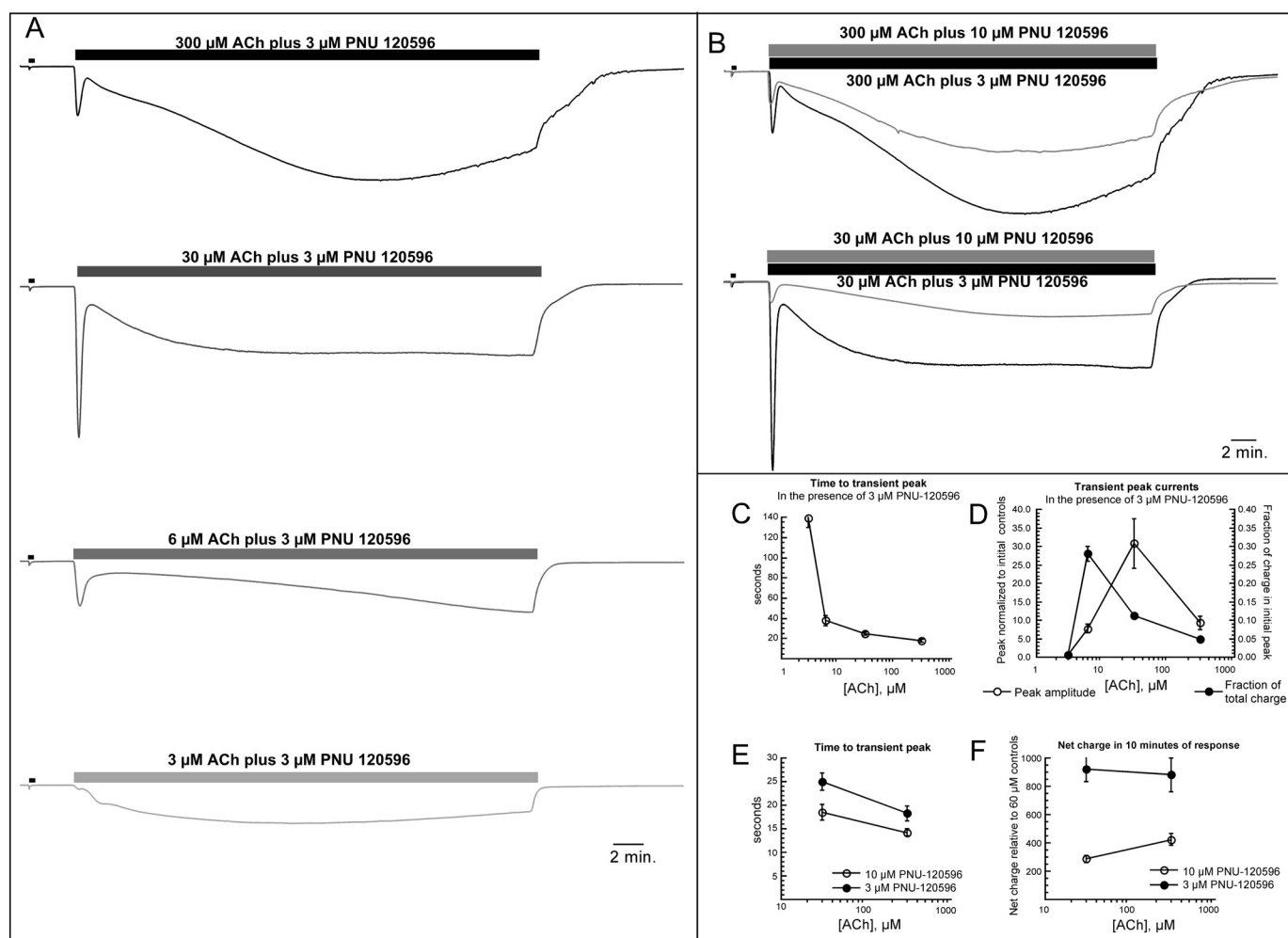


Fig. 10. Dynamic aspects of $\alpha 7$ nAChR responses to bath application of ACh and PNU-120596. A, representative traces illustrating the response of human $\alpha 7$ nAChR expressed in *X. laevis* oocytes to the bath application of 3 μ M PNU-120596 and various concentration of ACh, as indicated. All responses are scaled relative to each cell's response to a control application of 60 μ M ACh (black bar, far left) before 30-min bath applications of ACh and the PAM. B, representative responses to ACh coapplied with either 3 or 10 μ M PNU-120596. The data for the coapplication responses to 10 μ M PNU-120596 plus either 30 or 300 μ M ACh (gray) are superimposed over the responses to 3 μ M PNU-120596 plus either 30 or 300 μ M ACh (black), reproduced from A. All responses are scaled relative to each cell's response to a control application of 60 μ M ACh (black bar, far left). C, time to transient peak current recorded with the coapplication of 3 μ M PNU-120596 and varying concentrations of ACh. D, the amplitude of the transient peak currents (\circ and y-axis on left) at varying concentrations of ACh in 3 μ M PNU-120596 and the fraction of the total net charge in the first 10 min of the responses that accumulated during the transient peak (\bullet and y-axis on right). E, time to transient peak current recorded in the currents shown in B. F, the net charge in the first 10 min of responses obtained under the conditions illustrated in B.

decline by the end of the 30-min application, whereas the response evoked by 6 μ M ACh and 3 μ M PNU-120596 was still rising after 30 min.

After just 10 min of coapplication, the steady-state currents measured in the experiments shown in Fig. 10 were on average $6.5 \pm 0.8 \mu$ A, representing approximately 10^6 simultaneously open receptors. Although in each separate experiment at least six cells survived for the first 10 min, many cells could not be held in voltage clamp for the entire 30-min protocol with such high holding currents. Therefore, we restricted the measurement of response features to the first 10 min of the responses to have six to eight observations for each condition. Although the transient peaks of the 6 μ M responses were lower than those of the 30 μ M responses, the net charge accumulated during the transient phases of the 6 μ M responses was a greater fraction of the total net charge in the first 10 min of the responses than that of any of the other conditions (Fig. 10D, right *y*-axis; $P < 0.001$).

Clearly the slow and fast phases of the responses obtained by ACh coapplications with 3 μ M PNU-120596 were differentially affected by ACh concentrations. The data presented in Fig. 5 suggest that the concentration of PNU-120596 will also be a factor such that sustained currents may be less readily obtained with higher concentrations of the PAM. Figure 10B shows comparisons between the responses evoked by 30 or 300 μ M ACh coapplied with either 3 or 10 μ M PNU-120596. The peaks in the transient phase of the responses occurred more rapidly in the presence of the higher concentration of PNU-120596 (Fig. 10E, $p < 0.05$), and the total net charge in the first 10 min of the responses was less with the higher concentration of the PAM (Fig. 10F, $p < 0.001$), suggesting fast induction and greater levels of PAM-insensitive desensitization with the higher concentration of PNU-120596.

Varying Concentration-Dependent Effects of Competitive Antagonists Modulate P_{open} of PNU-120596-Primed Receptors. Our data suggest that optimal steady-state activation of $\alpha 7$ nAChR by combinations of agonists and PNU-120596 will be achieved when there is submaximal occupancy of both the agonist and PAM binding sites. To test this hypothesis, we made applications of varying concentrations of the $\alpha 7$ -selective competitive antagonist MLA to receptors responding to bath application of 3 μ M PNU-120596 and different concentrations of the $\alpha 7$ -selective agonist choline. The applications of MLA would be expected to change the fractional occupancy of the agonist binding sites, and, indeed, the effects of the MLA applications were observed to vary as a function of the choline concentrations. At the highest concentration of choline [600 μ M, responses roughly equivalent to 60 μ M ACh (Papke and Porter-Papke, 2002)], applications of both 10 nM and 100 nM MLA were observed to actually produce transient increases in the current during the slow phase of the responses, and the 1 μ M application produced a relatively small decrease in current (with a positive rebound). These effects are compared in Fig. 11 to the effect of a subsequent application of 100 μ M mecamylamine, a noncompetitive antagonist. These concentration-dependent effects of MLA shifted progressively when the choline concentrations were reduced. In the presence of 200 μ M choline, only the 10 nM MLA applications had purely positive effects, 100 nM had a biphasic effect, and the inhibition produced by 1 μ M MLA was more similar to that produced by 100 μ M

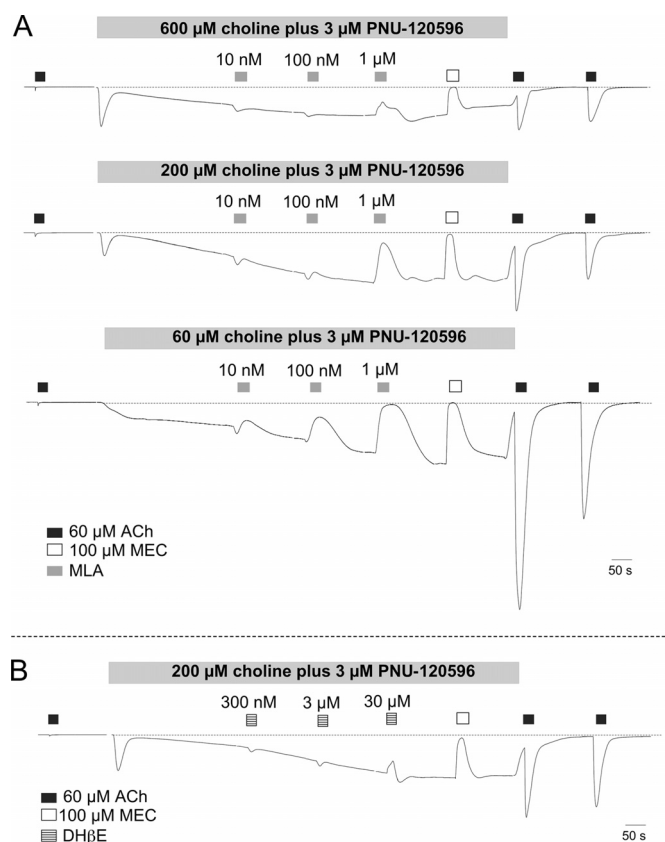


Fig. 11. Competitive antagonists are active molecules in modulating P_{open} of PNU-primed receptors. A, human $\alpha 7$ nAChR expressed in *X. laevis* oocytes were activated by bath coapplication of 3 μ M PNU-120596 and various concentrations of choline as indicated. Various concentrations of MLA were applied during the coapplication responses. As shown in the representative traces, the concentration dependence for MLA effects depended on the concentration of the agonist. In each experiment, the MLA applications were followed up by an application of 100 μ M the noncompetitive antagonist mecamylamine to confirm that the large currents were receptor-mediated. B, effects of the competitive antagonist DH β E on the responses of human $\alpha 7$ nAChR expressed in *X. laevis* oocytes to coapplication of 3 μ M PNU-120596 and 200 μ M choline. The effects of DH β E were similar to those of MLA, with a correction for the reduced potency of DH β E compared with MLA for the inhibition of $\alpha 7$ nAChR.

mecamylamine. Consistent with this pattern, in the presence of 60 μ M choline, 10 nM MLA had biphasic effects, and higher concentrations were purely inhibitory, with 1 μ M fully as effective as 100 μ M mecamylamine. The effect of MLA applications were confirmed to rely on $\alpha 7$ receptor activation as a result of the combination of agonist and PNU-120596, because MLA application had no detectable effect on cells expressing $\alpha 7$ and perfused with 3 μ M PNU-120596 alone (not shown). It was also confirmed that the effects of different MLA concentrations were independent of the order in which they were applied (not shown).

MLA is a large molecule, possibly capable of interacting with $\alpha 7$ nAChR in multiple ways. To confirm that the effects illustrated in Fig. 11A were through MLA's activity as a competitive antagonist, we tested the effect of dihydro- β -erythroidine (DH β E), a competitive antagonist with selectivity for $\alpha 4$ -containing nAChR but also active on $\alpha 7$ with an IC_{50} of approximately 8 μ M (Papke et al., 2008). As shown in Fig. 11B, applications of the two lower concentrations of DH β E produced transient increases in the currents that were

activated by 200 μ M choline in combination with 3 μ M PNU-120596, whereas the highest concentration of DH β E had a biphasic effect.

Note that in the oocyte system, the effects of priming the receptors with PNU-120596 applications are reversed relatively slowly (Papke et al., 2009), presumably as a result of delayed dissociation of the PAM from its binding sites. Therefore, in the experiments illustrated in Fig. 11, follow-up control applications of ACh were much larger than the initial ACh controls. The amplitudes of those responses were strongly influenced by the choline concentration in the preceding bath applications, further indicating that the higher choline concentrations promoted more PAM-insensitive desensitization.

Specific Ligands Differentially Regulate the Stability Receptors in the D_s and D_i States. Although full and partial agonists differ in their effectiveness for ion channel activation, when applied at high concentrations for long periods of time, both types of drugs desensitize $\alpha 7$ nAChR so that they are unable to be activated by a further application of a full agonist. Because our data indicate that there are at least two different $\alpha 7$ desensitized states that can be distinguished based on their sensitivity to PNU-120596, we determined whether specific ligands selectively promoted the stability of either the D_s or D_i forms of the receptor. Specifically, we compared ACh with three $\alpha 7$ -selective benzylidene anabaseine partial agonists, the structurally unrelated $\alpha 7$ -selective agonist tropisetron, and the very weak $\alpha 7$ partial agonist lobeline (Table 2). We applied these compounds for 5 min at relatively high effective concentrations (Papke et al., 2005, 2009) to two sets of oocytes expressing human $\alpha 7$ nAChR. We recorded varying levels of transient current during the initial moments of the drug application (Table 2, evoked response), and in all cases, these currents declined to baseline by the end of the 5-min incubation. One set of cells was then given an additional 1 mM ACh application to determine whether the receptors were fully desensitized. Responses to this follow-up application of ACh were only detected if the cells had been incubated with anabaseine or lobeline (Table 2, ACh residual); for all other treatments, desensitization seemed to be complete. To determine the relative expression of D_s and

D_i , the second set of cells received an application of 300 μ M PNU-120596 in the drug-containing incubation solution. The PNU-120596-evoked responses varied greatly and were not correlated to the apparent efficacy of the compounds on initial application (Table 2, PAM-evoked). Of the compounds that were fully desensitizing at the end of the 5-min incubations, ACh and tropisetron were most effective at placing the receptors in the D_s state, based on the subsequent responses to the drugs plus PNU-120596. The benzylidene anabaseine compounds, which were all of comparable efficacy in the initial phase of the incubation and equally desensitizing after 5 min, differed by over a factor of 10 in the induction of D_s . These molecules differ structurally only in the disposition of hydroxyl or methoxy side groups on the benzylidene ring, a portion of the molecule associated with $\alpha 7$ selectivity but located a considerable distance from the cationic core pharmacophore (Papke et al., 2009).

Energy Landscape Models Provide a Qualitative Description of $\alpha 7$ Activation and Desensitization. Basic models of nAChRs are required to account for closed states (C), open states (O), and desensitized states (D) and integrate the effects of agonist binding and the relative occupancy of these states. The model in Fig. 12 describes the receptor's functional states based on hypothetical relative free energy levels and intervening energy barriers through a matrix of increasing occupancies at both agonist and PAM binding sites. The relative energy levels and heights of the energy barriers are proportionate to the logs of the transition rate constants in standard Markov models. The model is minimal and omits potential intermediate states, such as the hypothetical flip state (Lape et al., 2008), but effectively represents qualitative characteristics of our data. Vertical distances represent absolute differences in free energy, or stability, and will be predictive of the relative occupancy of the various states under equilibrium conditions. To simplify comparisons between the schematics for the various levels of agonist occupancy, the resting closed states were set to the same level. However, it should be appreciated that the average free energy of the resting bound state for each schematic probably increases (becomes less stable) in a stepwise manner as agonist occupancy increases. In addition, it should be noted that the hypothetical energy landscapes in Fig. 12 are designed to explain our data for the full agonists ACh and choline, and the energy surfaces for partial agonists would probably be quite different (Table 2).

An instructive way to interpret this model is to imagine all of the receptors initially in the resting closed state at the moment of a change in agonist and/or PAM concentration, which will progressively take the receptors through the various levels of agonist occupancy. The top row of schematics illustrates the energy landscape and state transitions for $\alpha 7$ nAChR at various levels of ACh binding in the absence of PNU-120596. Transition from C to the short-lived open-state characteristic of $\alpha 7$ (O^*) will never occur at high probability but will be most likely to occur before saturation of the agonist binding sites. The energy well for the O^* open state in the model is very shallow, consistent with the brief and isolated openings recorded in the outside-out patch experiments (Fig. 2). The stability of the D_s and D_i states relative to C increases with agonist occupancy, and the equilibrium between D_s and D_i will most strongly favor D_i at the highest levels of agonist and PAM occupancy.

TABLE 2

Induction of D_s by 5-min incubations

Data obtained from *X. laevis* oocytes. All evoked responses are net charge values, normalized to the average net charge in two initial 60 μ M ACh applications. " D_s Induction" refers to rank order for the relative effectiveness of each drug for stabilizing receptors in the D_s state as measured by the ability of PNU-120596 application to stimulate currents of the predesensitized receptors.

Compound	Evoked Response	ACh Residual	PAM-Evoked	D_s Induction
ACh, 1 mM	1.12 \pm 0.08	0.01 \pm 0.01	32.1 \pm 2.3	1
diOHBA, 100 μ M	0.78 \pm 0.06	0.01 \pm 0.01	13.3 \pm 1.56	3
4OH2MeOBA, 100 μ M	0.61 \pm 0.03	0.02 \pm 0.01	0.95 \pm 0.15	5
Tropisetron, 10 μ M	0.61 \pm 0.03	0.01 \pm 0.01	25.6 \pm 2.2	2
diMeOBA, 100 μ M	0.52 \pm 0.17	0.03 \pm 0.03	2.97 \pm 0.38	4
Lobeline, 100 μ M	0.07 \pm 0.02	0.21 \pm 0.06	0.13 \pm 0.02	6

diOHBA, 3-(2,4-dihydroxybenzylidene)anabaseine; 4OH2MeOBA, 3-(4-hydroxy, 2-methoxybenzylidene)anabaseine; tropisetron, (1*R*,5*S*)-8-methyl-8-azabicyclo[3.2.0.1]octan-3-yl-1-methyl-indole-3-carboxylate; diOHBA, 3-(2,4-dihydroxybenzylidene)anabaseine; diMeOBA, 3-(2,4-dimethoxybenzylidene)anabaseine; lobeline, 2-[6-(2-hydroxy-2-phenylethyl)-1-methyl-2-piperidyl]-1-phenyl-ethanone.

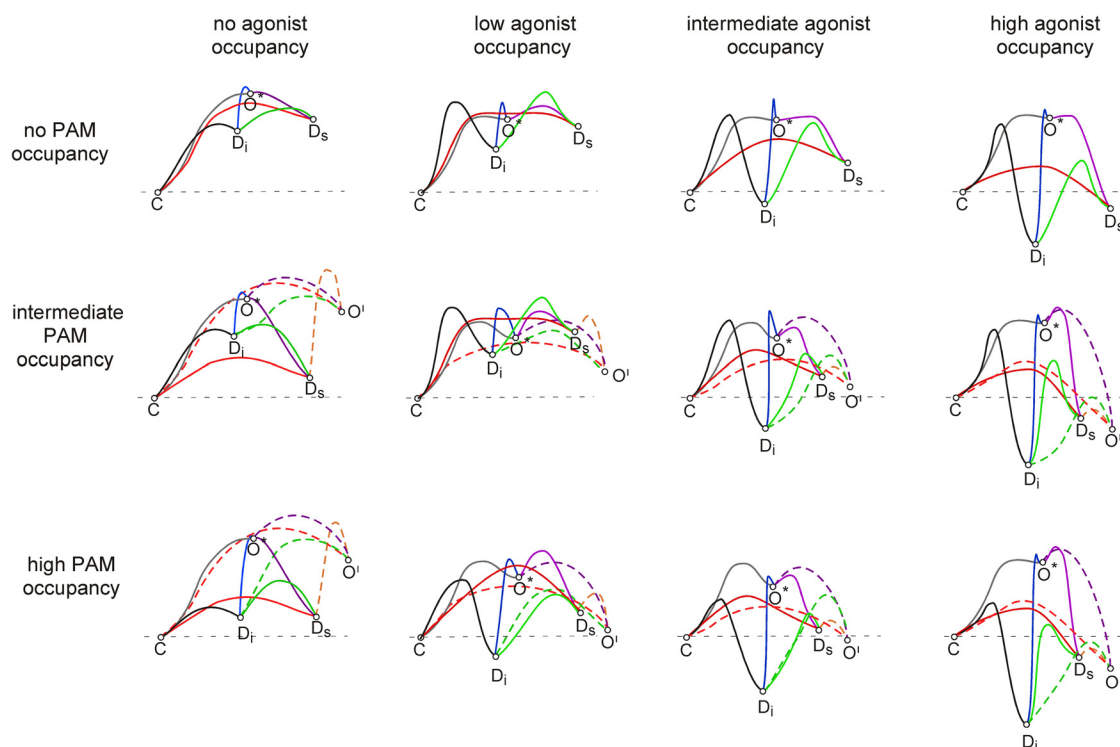
$\alpha 7$ -nAChR

Fig. 12. Proposed models for the activation, desensitization, and modulation of $\alpha 7$ nAChR. Shown are qualitative representations of minimal models for $\alpha 7$ nAChR conformational states, their relative energy levels, and transition rates as deduced from our data for varying levels of agonist and PNU-120596 binding. Under equilibrium conditions, the distributions of receptors into the resting closed, open, and desensitized states will be determined by the relative free energy of the states (represented by vertical displacements). The dynamic transition rates between the states will be inversely related to the log of the energy barriers between the states. In the absence of any PNU-120596 binding, the primary effect of agonist binding is to shift the equilibrium between the conformational states from the resting closed (C) state toward the desensitized states, D_s , which is sensitive to destabilization by PNU-120596, and D_i , which is insensitive to the activating effects of PNU-120596. The shallow energy well assigned to the open state (O^*) is consistent with the brief opening observed in single-channel recordings, and the high-energy barriers into the O^* state are consistent with the low P_{open} observed. The lower two rows include a new long-lived open state O' , promoted by the binding of PNU-120596 and possibly connected to a modified form of the D_s state, which is converted to a subconductance.

Single-channel openings evoked by ACh in the presence of PNU-120596 can occur as extremely long bursts or groups of openings separated by very short closures. This type of activity can be associated with the existence of a long-lived open state(s) (O'). We believe the D_s state represents the rapid concentration-dependent form of desensitization unique to $\alpha 7$, and we hypothesize that PNU-120596 converts or connects the D_s state into the O' state(s). Note that for simplicity, only one O' state is represented in the model; however, our burst analysis suggests that the O' states may be kinetically distinct at different levels of agonist and PAM occupancy. We hypothesize that the short intraburst closures, many of which appear as subconductances, represent the reverberation between D_s and O' in the presence of PNU-120596. The O' state(s) may be analogous to the long-lived open states of doubly liganded heteromeric receptors so that conversions between D_s and O' are the primary mechanism through which PNU-120596 reconciles the peak current and net charge ACh concentration-response curves of $\alpha 7$ receptors (Fig. 1). Entry into D_i states primarily occurs through activated channel states, and the barrier heights between O'/D_s and D_i states are reduced at high occupancy levels. Therefore, strong ion channel activation promotes D_i , and a primary effect of high PAM occupancy is to stabilize D_i . The stabilization of D_i by high levels of both agonist and PAM occupancy results in a window in which maximal poten-

tiation occurs within the low-intermediate range of both agonist and PAM occupancy.

Discussion

The initial characterizations of the type II PAMs PNU-120596, 3*a*,4,5,9*b*-tetrahydro-4-(1-naphthalenyl)-3*H*-cyclopentan[c]quinoline-8-sulfonamide (TQS), and 4-(5-(4-chlorophenyl)-2-methyl-3-propionyl-1*H*-pyrrol-1-yl)benzenesulfonamide (A-867744) reported that these agents reverse or eliminate desensitization (Williams et al., 2011b). Our results show that at least two distinct forms of $\alpha 7$ desensitization exist based on their stability in the presence of PNU-120596. The published literature lacks a formal description of D_i , but there are some published observations from other research groups that we interpret as consistent with the existence of PAM-insensitive D_i states. First, when agonist concentrations are increased in the presence of a fixed PAM concentration, responses are generally enhanced over the full agonist concentration ranges, but the magnitude of potentiation often tends to peak at intermediate concentrations and actually decrease at the higher agonist concentrations (Hurst et al., 2005; Grønlien et al., 2007; Dinklo et al., 2011). A similar phenomenon is sometimes observed when modulator concentrations are increased in the presence of a constant agonist concentration (Dunlop et al., 2009; Dinklo et

al., 2011). Second, applications of TQS (Grønlien et al., 2007) or A-867744 (Malysz et al., 2009) were shown to reactivate receptors that had been desensitized by a high concentration of agonist and produced a nondecaying current on the time scale of the experiments, consistent with reversal of D_s states to O' states. However, the peak of the potentiated current in the presence of either TQS or A-867744 was only $\sim 50\%$ of the peak response recorded in the absence of PAM, indicating that only a fraction of the receptor populations were actually conducting current at any given moment. Third, several applications of $30\ \mu\text{M}$ nicotine, with short interstimulus intervals, in the continued presence of PNU-120596 produced successively smaller responses (Ng et al., 2007), indicative of the accumulation of modulator-insensitive D_i states. Fourth, an α -bungarotoxin-insensitive and PNU-120596-dependent increase in calcium response from bovine chromaffin cells was only observed when low ($1\ \mu\text{M}$) concentrations of nicotine were used to stimulate the cells. At concentrations of $\geq 3\ \mu\text{M}$ nicotine, neither PNU-120596 nor α -bungarotoxin had an effect on the response, indicating that the $\alpha 7$ component was lost by the higher nicotine concentrations (del Barrio et al., 2011). In addition, when the $\alpha 7$ component of the total calcium response was isolated, increased concentrations of the $\alpha 7$ agonist *N*-(3*R*)-1-azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide (PNU-282987) with a fixed PNU-120596 concentration resulted in decreased $\alpha 7$ -dependent calcium responses (del Barrio et al., 2011).

The truly dynamic nature of the interacting effects of agonist and PNU-120596 over time are illustrated by the long bath application responses showing biphasic currents. Biphasic currents have also been reported in currents potentiated by TQS (Grønlien et al., 2007; Malysz et al., 2009). There are numerous factors contributing to these responses, which are difficult to tease apart. The receptors are initially assumed to be in the unliganded resting closed state, with agonist binding occurring as functions of the solution exchange rate and the agonist concentration applied. Our data suggest that PNU-120596 binding occurs more slowly than ACh binding but is cooperatively influenced by channel activation because the onset of PNU-120596 potentiation occurs more rapidly with strong receptor stimulation by agonist. It is noteworthy that the transient peak currents are smaller and occur faster with increasing concentrations of ACh and PNU-120596, suggesting that a limiting process is increased by both of these factors (Papke, 2010). It seems reasonable to hypothesize that this limiting factor is D_i . We further hypothesize that an immediate effect of PNU-120596 may be to lower the energy barriers for agonist-evoked activation, with relatively little initial effect on desensitization, and that the steady-state currents develop over time as PNU-120596 converts D_s states into O' states and alters the relative free energy levels and establishes a new equilibrium between nonconducting D_i and conducting D_s/O' states. Interestingly, our data suggest that competitive antagonists may influence the equilibrium established between D_s and D_i during prolonged agonist and PNU-120596 applications to produce either positive or negative effects, depending on agonist concentration. It is noteworthy that competitive antagonists seemed to have the effect of shifting the equilibrium in favor of D_s and activatable states when agonist concentration and, by inference, agonist occupancy was high.

The strong apparent induction of D_i in the outside-out patches, yet with extremely long bursting activity of individ-

ual channels, suggests PNU-120596 is capable of having very large effects on just a few channels at a time. Although the net charge of an oocyte whole-cell current is increased as much as 500-fold by PNU-120596, the net charge of a single-channel burst for a PNU-120596-primed channel is approximately 10^5 times greater than that of a nonpotentiated $\alpha 7$ single-channel opening. These two numbers are resolved if only 1 in 200 receptors is active in the bursting states at any moment during a PNU-120596 treatment. Although our data suggest that high levels of agonist occupancy generally disfavor channel opening through stabilization of D_i , the mode by which a few resilient PNU-120596-primed channels escape D_i and enter the hyperbursting state, despite high agonist and PAM occupancy, is less clear. These observations indicate that PNU-120596-primed currents should be used with caution for setting a lower-limit on N , meaning the $\alpha 7$ P_{open} estimate after application of $60\ \mu\text{M}$ ACh could be much less than 7.4×10^{-6} . Even if this upper limit is greatly overestimated, the data in Fig. 2 argue strongly against the assumption that $\alpha 7$ P_{open} is high immediately after rapid exposure to agonist, as proposed previously (Bouzat et al., 2008).

The observation that bursting activity of single-channels in the presence of PNU-120596 persists for approximately 2.5 s after removal of external ACh is consistent with the prolonged retention of ACh once channels enter the PNU-120596-primed bursting mode. This observation, in combination with the finding that the onset of potentiation by PNU-120596 is dependent on channel activity, suggests the possibility that agonist and PAM binding sites mutually interact. This possibility is supported by a recent report that the potency of the agonist *N*-(3*R*)-1-azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide (PNU-282987) to evoke calcium responses in IMR-32 cells was increased with higher PNU-120596 concentrations; vice versa, the potency of PNU-120596 to potentiate responses was increased with higher PNU-282987 concentrations (Gopalakrishnan et al., 2011). In addition, computer docking simulations predicted that binding of PNU-120596 to the proposed transmembrane cavity has higher affinity in an "open" channel model than in a "closed" channel model (Young et al., 2008).

Activation of $\alpha 7$ can have either protective or toxic effects depending on the mode of stimulation (Li et al., 1999), and the potential for enormous potentiation of $\alpha 7$ responses by type II PAMs invites the concern that potentiation of $\alpha 7$ with type II PAMs may bring receptor activation to dangerously high levels (for review, see Williams et al., 2011b). There are contradicting reports in the published literature regarding the in vitro cytotoxicity profile of PNU-120596; in the two cases where PNU-120596 was found to be toxic (Ng et al., 2007; Dinklo et al., 2011), the stimulating agonist was the weakly potent $\alpha 7$ agonist choline at a concentration of $\sim 100\ \mu\text{M}$, which is well below the published EC_{50} value (Papke and Porter-Papke, 2002) and potentially falls within the window of strong $\alpha 7$ potentiation where D_i is avoided. In contrast, PNU-120596 was reported to lack a significant cytotoxicity profile when coapplied with a $10\ \mu\text{M}$ concentration of the potent $\alpha 7$ agonist PNU-282987 (Hu et al., 2009). Given that the published EC_{50} value of PNU-282987 in the presence of $3\ \mu\text{M}$ PNU-120596 is $\sim 200\ \text{nM}$ (Grønlien et al., 2007), and D_i induced by high PNU-282987 concentrations is apparent in bovine chromaffin cells (del Barrio et al., 2011), the existence

of D_i states, which may provide an intrinsic safety mechanism, might account for the lack of toxicity in this study and for the discrepancy in the published literature regarding type II PAM toxicity profiles.

Our data indicate that even for the structurally related benzylidene anabaseine compounds, there are significant equilibrium energy differences associated with the D_s and D_i states. Molecular docking studies of the benzylidene anabaseine compounds suggest that the benzylidene group extends into the intersubunit interface (Horenstein et al., 2007), and our data would suggest that specific point-to-point interactions between sidegroups on the benzylidene and specific amino acid residues in the cleft may be exploited to differentially stabilize receptors in the D_s and D_i states. The structure of the acetylcholine binding protein bound with 3-(2,4-dimethoxybenzylidene)anabaseine, the compound that most effectively induced D_i or other nonresponsive states, showed that the molecule might be bound in entirely different orientations at the various subunit interfaces (Hibbs et al., 2009). In addition, recent work with tethered agonists has shown that a single ligand molecule may bind the ligand binding domain in different orientations, each of which may differentially affect activation and desensitization (Wang et al., 2010). Identification of compounds that preferentially stabilize specific functional states, including D_s and D_i , may be therapeutically important if it can be shown that a specific state is important for signal transduction attributed to $\alpha 7$. It is noteworthy that $\alpha 7$ -mediated signal transduction has been observed under conditions in which ion channel activation is not apparent (Skok, 2009), leaving open the intriguing possibility that $\alpha 7$ -desensitized states are not inactive but functionally meaningful.

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Authorship Contributions

Participated in research design: Williams and Papke.
Conducted experiments: Williams.
Contributed new reagents or analytic tools: Wang.
Performed data analysis: Williams and Papke.
Wrote or contributed to the writing of the manuscript: Williams, Wang, and Papke.

References

- Bertrand S, Devillers-Thiery A, Palma E, Buisson B, Edelstein SJ, Corringer PJ, Changeux JP, and Bertrand D (1997) Paradoxical allosteric effects of competitive inhibitors on neuronal $\alpha 7$ nicotinic receptor mutants. *Neuroreport* **8**:3591–3596.
- Bouzat C, Bartos M, Corradi J, and Sine SM (2008) The interface between extracellular and transmembrane domains of homomeric Cys-loop receptors governs open-channel lifetime and rate of desensitization. *J Neurosci* **28**:7808–7819.
- Colquhoun D and Sakmann B (1985) Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. *J Physiol (Lond)* **369**:501–557.
- Colquhoun D and Sakmann B (1995) Fitting and statistical analysis of single-channel records, in *Single-Channel Recording* (Sakmann B and Neher E eds) pp 483–585. Plenum Press, New York.
- del Barrio L, Egea J, León R, Romero A, Ruiz A, Montero M, Alvarez J, and López MG (2011) Calcium signalling mediated through $\alpha 7$ and non- $\alpha 7$ nAChR stimulation is differentially regulated in bovine chromaffin cells to induce catecholamine release. *Br J Pharmacol* **162**:94–110.
- Dinklo T, Shaban H, Thuring JW, Lavreysen H, Stevens KE, Zheng L, Mackie C, Grantham C, Vandenberk I, Meulders G, et al. (2011) Characterization of 2-[[4-fluoro-3-(trifluoromethyl)phenyl]amino]-4-(4-pyridinyl)-5-thiazolemethanol (JNJ-1930942), a novel positive allosteric modulator of the $\alpha 7$ nicotinic acetylcholine receptor. *J Pharmacol Exp Ther* **336**:560–574.
- Dunlop J, Lock T, Jow B, Sitzia F, Grauer S, Jow F, Kramer A, Bowlby MR, Randall A, Kowal D, et al. (2009) Old and new pharmacology: positive allosteric modulation of the $\alpha 7$ nicotinic acetylcholine receptor by the 5-hydroxytryptamine(2B/C) receptor antagonist SB-206553 (3,5-dihydro-5-methyl-N-3-pyridinylbenzo[1,2-b:4,5-b']di pyrrole-1(2H)-carboxamide). *J Pharmacol Exp Ther* **328**:766–776.
- Gopalakrishnan SM, Philip BM, Gronlien JH, Malysz J, Anderson DJ, Gopalakrishnan M, Warrior U, and Burns DJ (2011) Functional characterization and high-throughput screening of positive allosteric modulators of $\alpha 7$ nicotinic acetylcholine receptors in IMR-32 neuroblastoma cells. *Assay Drug Dev Technol* doi: 10.1089/adt.2010.0319.
- Grønlien JH, Håkerud M, Ween H, Thorin-Hagene K, Briggs CA, Gopalakrishnan M, and Malysz J (2007) Distinct profiles of $\alpha 7$ nAChR positive allosteric modulation revealed by structurally diverse chemotypes. *Mol Pharmacol* **72**:715–724.
- Halevi S, McKay J, Palfreyman M, Yassin L, Eshel M, Jorgensen E, and Treinin M (2002) The C. elegans ric-3 gene is required for maturation of nicotinic acetylcholine receptors. *EMBO J* **21**:1012–1020.
- Heidmann T and Changeux JP (1978) Structural and functional properties of the acetylcholine receptor protein in its purified and membrane-bound states. *Annu Rev Biochem* **47**:317–357.
- Hibbs RE, Sulzenbacher G, Shi J, Talley TT, Conrod S, Kem WR, Taylor P, Marchot P, and Bourne Y (2009) Structural determinants for interaction of partial agonists with acetylcholine binding protein and neuronal $\alpha 7$ nicotinic acetylcholine receptor. *EMBO J* **28**:3040–3051.
- Horenstein NA, McCormack TJ, Stokes C, Ren K, and Papke RL (2007) Reversal of agonist selectivity by mutations of conserved amino acids in the binding site of nicotinic acetylcholine receptors. *J Biol Chem* **282**:5899–5909.
- Hu M, Gopalakrishnan M, and Li J (2009) Positive allosteric modulation of $\alpha 7$ neuronal nicotinic acetylcholine receptors: lack of cytotoxicity in PC12 cells and rat primary cortical neurons. *Br J Pharmacol* **158**:1857–1864.
- Hurst RS, Hájós M, Raggenbass M, Wall TM, Higdon NR, Lawson JA, Rutherford-Root KL, Berkenpas MB, Hoffmann WE, Piotrowski DW, Groppi VE, Allaman G, Ogier R, Bertrand S, Bertrand D, and Arneric SP (2005) A novel positive allosteric modulator of the $\alpha 7$ neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization. *J Neurosci* **25**:4396–4405.
- Lape R, Colquhoun D, and Sivilotti LG (2008) On the nature of partial agonism in the nicotinic receptor superfamily. *Nature* **454**:722–727.
- Li Y, Papke RL, He YJ, Millard WJ, and Meyer EM (1999) Characterization of the neuroprotective and toxic effects of $\alpha 7$ nicotinic receptor activation in PC12 cells. *Brain Res* **830**:218–225.
- Malysz J, Grønlien JH, Anderson DJ, Håkerud M, Thorin-Hagene K, Ween H, Wetterstrand C, Briggs CA, Faghih R, Bunnelle WH, et al. (2009) In vitro pharmacological characterization of a novel allosteric modulator of $\alpha 7$ neuronal acetylcholine receptor, 4-(5-(4-chlorophenyl)-2-methyl-3-propionyl-1H-pyrrol-1-yl)benzenesulfonamide (A-867744), exhibiting unique pharmacological profile. *J Pharmacol Exp Ther* **330**:257–267.
- McNerney ME, Pardi D, Pugh PC, Nai Q, and Margiotta JF (2000) Expression and channel properties of $\alpha 7$ nicotinic acetylcholine receptors on chick ciliary and choroid neurons. *J Neurophysiol* **84**:1314–1329.
- Mike A, Castro NG, and Albuquerque EX (2000) Choline and acetylcholine have similar kinetic properties of activation and desensitization on the $\alpha 7$ nicotinic receptors in rat hippocampal neurons. *Brain Res* **882**:155–168.
- Millar NS and Gotti C (2009) Diversity of vertebrate nicotinic acetylcholine receptors. *Neuropharmacology* **56**:237–246.
- Ng HJ, Whittemore ER, Tran MB, Hogenkamp DJ, Broide RS, Johnstone TB, Zheng L, Stevens KE, and Gee KW (2007) Nootropic $\alpha 7$ nicotinic receptor allosteric modulator derived from GABAA receptor modulators. *Proc Natl Acad Sci USA* **104**:8059–8064.
- Palma E, Bertrand S, Binzoni T, and Bertrand D (1996) Neuronal nicotinic $\alpha 7$ receptor expressed in *Xenopus* oocytes presents five putative binding sites for methyllycaconitine. *J Physiol* **491**:151–161.
- Papke RL, Craig AG, and Heinemann SF (1994) Inhibition of nicotinic acetylcholine receptors by bis (2,2,6,6-tetramethyl-4-piperidinyl) sebacate (Tinuvin 770), an additive to medical plastics. *J Pharmacol Exp Ther* **268**:718–726.
- Papke RL (2010) Tricks of perspective: insights and limitations to the study of macroscopic currents for the analysis of nAChR activation and desensitization. *J Mol Neurosci* **40**:77–86.
- Papke RL, Dwoskin LP, Crooks PA, Zheng G, Zhang Z, McIntosh JM, and Stokes C (2008) Extending the analysis of nicotinic receptor antagonists with the study of $\alpha 7$ nicotinic receptor subunit chimeras. *Neuropharmacology* **54**:1189–1200.
- Papke RL, Kem WR, Soti F, López-Hernández GY, and Horenstein NA (2009) Activation and desensitization of nicotinic $\alpha 7$ -type acetylcholine receptors by benzylidene anabaseines and nicotine. *J Pharmacol Exp Ther* **329**:791–807.
- Papke RL, Meyer E, Nutter T, and Uteshev VV (2000) $\alpha 7$ -selective agonists and modes of $\alpha 7$ receptor activation. *Eur J Pharmacol* **393**:179–195.
- Papke RL and Porter Papke JK (2002) Comparative pharmacology of rat and human $\alpha 7$ nAChR conducted with net charge analysis. *Br J Pharmacol* **137**:49–61.
- Papke RL, Schiff HC, Jack BA, and Horenstein NA (2005) Molecular dissection of tropisetron, an $\alpha 7$ nicotinic acetylcholine receptor-selective partial agonist. *Neurosci Lett* **378**:140–144.
- Papke RL and Stokes C (2010) Working with OpusXpress: methods for high volume oocyte experiments. *Methods* **51**:121–133.
- Papke RL and Thinschmidt JS (1998) The correction of $\alpha 7$ nicotinic acetylcholine receptor concentration-response relationships in *Xenopus* oocytes. *Neurosci Lett* **256**:163–

166. Piotrowski DW, Rogers BN, McWhorter WW Jr, Walker DP, Corbett JW, Groppi VE Jr, and Rudmann DG (2003), inventors; Pharmacia & Upjohn Company, Piotrowski DW, Rogers BN, McWhorter WW Jr, Walker DP, Corbett JW, Groppi VE Jr, and Rudmann DG, assignees. Preparation of ureas as positive allosteric modulators of the nicotinic acetylcholine receptor. World patent WO/2003/093250. 2003 Nov 13. <http://www.wipo.int/patentscope/search/en/WO2003093250>
- Placzek AN, Grassi F, Meyer EM, and Papke RL (2005) An $\alpha 7$ nicotinic acetylcholine receptor gain-of-function mutant that retains pharmacological fidelity. *Mol Pharmacol* **68**:1863–1876.
- Qin F (2004) Restoration of single-channel currents using the segmental k-means method based on hidden Markov modeling. *Biophys J* **86**:1488–1501.
- Skok MV (2009) To channel or not to channel? Functioning of nicotinic acetylcholine receptors in leukocytes. *J Leukoc Biol* **86**:1–3.
- Wang J, Horenstein NA, Stokes C, and Papke RL (2010) Tethered agonist analogs as site-specific probes for domains of the human $\alpha 7$ nicotinic acetylcholine receptor that differentially regulate activation and desensitization. *Mol Pharmacol* **78**:1012–1025.
- Williams DK, Stokes C, Horenstein NA, and Papke RL (2011a) The effective opening

of nicotinic acetylcholine receptors with single agonist binding sites. *J Gen Physiol* **137**:369–384.

- Williams DK, Wang J, and Papke RL (2011b) Positive allosteric modulators as an approach to nicotinic acetylcholine receptor-targeted therapeutics: Advantages and limitations. *Biochem Pharmacol* **82**:915–930.
- Young GT, Zwart R, Walker AS, Sher E, and Millar NS (2008) Potentiation of $\alpha 7$ nicotinic acetylcholine receptors via an allosteric transmembrane site. *Proc Natl Acad Sci USA* **105**:14686–14691.
- Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J, and Lindstrom J (2003) Human $\alpha 4\beta 2$ acetylcholine receptors formed from linked subunits. *J Neurosci* **23**:9004–9015.

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