

## $\alpha$ -Conotoxins PnIA and [A10L]PnIA Stabilize Different States of the $\alpha$ 7-L247T Nicotinic Acetylcholine Receptor\*

Received for publication, December 11, 2002, and in revised form, April 28, 2003  
Published, JBC Papers in Press, May 13, 2003, DOI 10.1074/jbc.M212628200

Ron C. Hogg<sup>‡</sup>, Gene Hopping<sup>§</sup>, Paul F. Alewood<sup>§</sup>, David J. Adams<sup>§</sup>, and Daniel Bertrand

From the Department of Physiology, CMU, 1 rue Michel Servet, CH-1211 Geneva 4, Switzerland and the <sup>§</sup>School of Biomedical Sciences, University of Queensland, Brisbane 4072, Australia

The effects of the native  $\alpha$ -conotoxin PnIA, its synthetic derivative [A10L]PnIA and alanine scan derivatives of [A10L]PnIA were investigated on chick wild type  $\alpha$ 7 and  $\alpha$ 7-L247T mutant nicotinic acetylcholine receptors (nAChRs) expressed in *Xenopus* oocytes. PnIA and [A10L]PnIA inhibited acetylcholine (ACh)-activated currents at wt $\alpha$ 7 receptors with IC<sub>50</sub> values of 349 and 168 nM, respectively. Rates of onset of inhibition were similar for PnIA and [A10L]PnIA; however, the rate of recovery was slower for [A10L]PnIA, indicating that the increased potency of [A10L]PnIA at  $\alpha$ 7 receptors is conveyed by its slower rate of dissociation from the receptors. All the alanine mutants of [A10L]PnIA inhibited ACh-activated currents at wt $\alpha$ 7 receptors. Insertion of an alanine residue between position 5 and 13 and at position 15 significantly reduced the ability of [A10L]PnIA to inhibit ACh-evoked currents. PnIA inhibited the non-desensitizing ACh-activated currents at  $\alpha$ 7-L247T receptors with an IC<sub>50</sub> 194 nM. In contrast, [A10L]PnIA and the alanine mutants potentiated the ACh-activated current  $\alpha$ 7-L247T receptors and in addition [A10L]PnIA acted as an agonist. PnIA stabilized the receptor in a state that is non-conducting in both the wild type and mutant receptors, whereas [A10L]PnIA stabilized a state that is non-conducting in the wild type receptor and conducting in the  $\alpha$ 7-L247T mutant. These data indicate that the change of a single amino acid side-chain, at position 10, is sufficient to change the toxin specificity for receptor states in the  $\alpha$ 7-L247T mutant.

Conotoxins are short peptides isolated from the venom of predatory marine snails of the genus *Conus*. Many of these toxins are selective inhibitors of ligand- and voltage-gated ion channels and are classified according to the type of channel to which they bind. This high selectivity of the conotoxins has led to a great interest in the use of these molecules as pharmacological tools and the design of novel therapeutics. Much of this work has been summarized in several recent reviews (1, 2). The  $\alpha$ -conotoxins are between 11 and 16 amino acids in length and are selective inhibitors of nicotinic acetylcholine receptors (nAChRs),<sup>1</sup> see Refs. 3 and 4 for review. Native nAChRs are composed of a number of distinct subunits, which combine to

form functional receptors each with distinct pharmacological properties. The  $\alpha$ -conotoxins contain 4 cysteines, which in their natural conformation form disulphide bridges giving the molecule a globular two-loop configuration with sidechains projecting from a rigid backbone. The  $\alpha$ -conotoxin PnIA, isolated from the molluscivorous cone snail *Conus pennaceus*, is 16 amino acids long, and the x-ray crystal structure shows an  $\alpha$ -helix between residues 5 and 12 and a 3<sub>10</sub> helical turn at the N-terminal end (5). PnIA has been demonstrated to be a competitive inhibitor of native nAChRs in cultured *Aplysia* neurons (6), dissociated neurons from rat parasympathetic ganglia (7), and recombinant nAChRs expressed in *Xenopus* oocytes (8) with poor selectivity between receptor subtypes. A leucine for alanine substitution at position 10 makes the toxin a highly selective inhibitor of the  $\alpha$ 7 nAChR subtype (7, 8), see Table I. Since the A10L mutation in PnIA changes the selectivity of the toxin for receptor subtypes, it is also possible that the mechanism of inhibition differs between PnIA and [A10L]PnIA, the A10L mutation may cause the toxins to have different affinities for different states of the receptor.

The nAChRs have been presented as a prototype of allosteric membrane protein (9) as described by the Monod-Wyman-Changeux model of allosteric interactions (10) in which the structure of the molecule moves in concerted transitions between pre-existing conformational states. The protein can exist in different states and undergoes spontaneous conformational transitions, in the absence of a ligand the equilibrium between these conformational states is in favor of the resting (closed) state. Exposure to an agonist preferentially stabilizes the receptor in the active (open) and desensitized (closed) states, whereas the binding of an antagonist molecule binds to and stabilizes the molecule in a closed (resting or desensitized) state (see Ref. 11). In such a model, transition from one state to another depends upon both the presence of a ligand and/or the isomerization coefficient, which governs changes between states. Binding of a molecule at a site distinct from the agonist-binding site may modify the isomerization coefficient, thus affecting agonist or antagonist behavior. Molecules acting in this way are known as allosteric effectors (10).

To test whether mutations of PnIA affected the inhibitory mechanism of the toxin, we examined the effects of PnIA, [A10L]PnIA and alanine scan mutants of [A10L]PnIA at wild type homomeric chick  $\alpha$ 7 receptors (wt $\alpha$ 7) and at chick  $\alpha$ 7 receptors with the L247T mutation ( $\alpha$ 7-L247T) expressed in *Xenopus* oocytes.  $\alpha$ 7-L247T receptors display non-desensitizing currents in response to agonist application (12–16). Because it was proposed that the L247T mutation renders conductive one of the desensitized states (12), this mutant receptor can be used as a tool to determine whether the same closed state of the

tetraacetic acid acetoxymethyl ester; HPLC, high performance liquid chromatography; MLA, methyllycaconitine.

\* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>‡</sup> To whom correspondence should be addressed: Dept. of Physiology, C.M.U. 1, rue Michel Servet, CH-1211 Geneva 4, Switzerland. Tel.: 41-22-702-53-55; Fax: 41-22-702-54-02; E-mail: hogg1@etu.unige.ch.

<sup>1</sup> The abbreviations used are: ACh, acetylcholine; nAChR, nicotinic ACh receptor; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N',N'*-

TABLE I  
Sequences of  $\alpha$ -conotoxins PnIA and [A10L]PnIA

Name	Sequence
PnIA	G <span style="border: 1px solid black; padding: 2px;">CC</span> S L P P <span style="border: 1px solid black; padding: 2px;">C</span> A A N N P D Y <span style="border: 1px solid black; padding: 2px;">C</span> -NH <sub>2</sub>
[A10L]PnIA	G <span style="border: 1px solid black; padding: 2px;">CC</span> S L P P <span style="border: 1px solid black; padding: 2px;">C</span> A L N N P D Y <span style="border: 1px solid black; padding: 2px;">C</span> -NH <sub>2</sub>

receptor is stabilized by PnIA and [A10L]PnIA, allowing us to probe states of the receptor which are otherwise electrophysiologically silent in the wild type receptor. To test the contribution of projecting side-chains at other positions to the activity of [A10L]PnIA, we have replaced each of the residues in turn with an alanine to observe the effects of this mutation on toxin activity. Circular dichroism spectra and <sup>1</sup>H NMR experiments have shown that substitution of individual residues in [A10L]PnIA with an alanine do not result in perturbation of the global fold,<sup>2</sup> thus, any changes in activity in these molecules can be directly correlated to the interactions of the projecting side chains with the nAChR.

#### EXPERIMENTAL PROCEDURES

**Electrophysiology**—*Xenopus* oocytes were prepared and injected as described previously (17). Two-electrode voltage clamp recordings were made 2–3 days after cDNA injection. During recordings the bath solution was OR2 medium containing (in mM), NaCl, 82.5; KCl, 2.5; CaCl<sub>2</sub>, 2.5; MgCl<sub>2</sub>, 1; atropine, 0.5; HEPES, 5, adjusted to pH 7.4 with NaOH. ACh was applied in a fast flowing solution stream (~6 ml/min). Unless indicated, oocytes were incubated in 100  $\mu$ M BAPTA-AM for at least 2 h prior to recording. Two-electrode voltage clamp recordings were carried out using a GeneClamp 500B amplifier (Axon Instruments Inc., Union City, CA), recording electrodes contained 3 mM KCl and oocytes were clamped at -100 mV throughout the experiments. Experiments were carried out at 18 °C. Dose-response curves for toxin inhibition were fit with the equation  $y = 1 / (1 + ([\text{toxin}] / \text{IC}_{50})^n)$ , where  $y$  is the normalized response, [toxin] is the toxin concentration, and  $n$  is the Hill coefficient. Measurements of rates of toxin block and recovery were carried out at toxin concentrations close to the  $\text{IC}_{50}$ . Data points for onset of block were fit with the equation  $y = A e^{-t/\tau} + B$ , where  $A$  = control amplitude normalized to 1,  $B$  = current amplitude following block expressed as a fraction of the control current, and  $t$  = time. Curves were fit using a  $\chi^2$  minimization fitting routine in Microcal origin 5.0 (Microcal software inc.). Data are presented as mean  $\pm$  S.E.

**Toxin Synthesis**—The 16-residue peptides were synthesized manually on a 0.50-mmol scale using HBTU activation of *t*-butoxycarbonyl-amino acids with *in situ* neutralization chemistry as described previously (18). The syntheses were performed on *p*-methylbenzhydrylamine resin using standard amino acid side chain protection. Each residue was coupled for 10 min and coupling efficiencies determined by the quantitative ninhydrin reaction (18). Prior to a standard HF cleavage (10 ml of *p*-cresol:*p*-thiocresol:HF 1:1:8 (0 °C, 2 h) and workup (18), the N-terminal *t*-butoxycarbonyl protecting group was removed (100% trifluoroacetic acid), and the resin was successively washed with dimethylformamide and dichloromethane. Air oxidations were carried out by dissolving the lyophilized purified A10L[PnIA] analogues in 0.1 M NH<sub>4</sub>HCO<sub>3</sub>/isopropyl alcohol (pH 8.25) with vigorous stirring at room temperature overnight. Prior to purification the solution was acidified to pH 3 with trifluoroacetic acid and analyzed by analytical C4 HPLC using a linear gradient of 0–80% solvent B at 1%/min while monitoring by UV absorbance at 214 nm and electrospray mass spectrometry. Oxidized material was then purified by semipreparative HPLC using the same chromatographic conditions.

#### RESULTS

**PnIA and [A10L]PnIA Inhibit ACh-induced Currents at Chick  $\alpha 7$  nAChR Receptors**—The effects of PnIA were investigated on chick  $\alpha 7$  nAChR receptors expressed in *Xenopus* oocytes. Membrane currents were activated with 200  $\mu$ M ACh, which is close to the  $\text{EC}_{50}$  (115  $\mu$ M) for these receptors (19).

PnIA inhibited ACh-induced currents in a concentration-dependent and reversible manner (Fig. 1A). The dose-response relationship is shown in Fig. 1B; the fitted curve had an  $\text{IC}_{50}$  of 349 nM and a slope of 1.8.

The position 10 mutant of PnIA, [A10L]PnIA, has previously been reported to inhibit ACh-induced currents at rat  $\alpha 7$  receptors expressed in *Xenopus* oocytes (8) and native  $\alpha 7$  receptors in rat parasympathetic ganglion neurons (7). [A10L]PnIA was twice as potent as PnIA to inhibit ACh-activated currents at chick  $\alpha 7$  receptors (Fig. 1C). Dose-response relationship was fit by a curve with an  $\text{IC}_{50}$  value of 168 nM and a slope of 1.55 (Fig. 1D).

**Rate of Block and Recovery**—The rate of onset and recovery from block of ACh-activated currents at  $\alpha 7$  receptors was measured for PnIA and [A10L]PnIA. As PnIA competes with ACh at the binding site, ACh was applied for 2 s, and it was assumed that these short applications of ACh did not displace the toxin. Toxin concentrations used were close to the respective  $\text{IC}_{50}$  values. The rate of block with PnIA (400 nM) followed an exponential time course with a  $\tau_{\text{onset}}$  of 11.3 s (Fig. 2A).

An exponential rate of block and recovery would suggest that the reaction is bimolecular. However, as the Hill coefficient for PnIA inhibition is greater than unity (Fig. 1B), more than one toxin binding site must be present. Since the homomeric  $\alpha 7$  receptor contains five putative agonist binding sites, we tried to fit the data using a scheme similar to MLA blockade of the  $\alpha 7$  receptor (20). If the occupation of a single binding site is sufficient to prevent activation of the receptor by an agonist, then the onset of block will follow an exponential time course. However, recovery from block requires that all of the binding sites are unoccupied, causing an initial lag in the recovery. Palma *et al.* (20) found that the recovery of block from MLA was best fit by a five-site model. Since the time course of recovery from MLA inhibition is ~10-fold slower than recovery from [A10L]PnIA and more than 20-fold slower than PnIA, this makes it likely that any lag in the recovery would not be resolved at our sampling interval of 1, 5, and 10 s. Thus the recovery describes only the dissociation of the last toxin molecule from the receptor. The time to half-recovery from PnIA inhibition was in the region of 15 s. The onset of block of [A10L]PnIA (200 nM) also followed an exponential time course with a  $\tau_{\text{onset}}$  of 12.0 s and had a slower half-recovery time in the region of 70 s (Fig. 3, C and D).

Fitting the data with a five site model (see Equation 3 from Ref. 20), using approximate rates of  $K_{\text{off}}$  and  $K_{\text{on}}$  from Fig. 2, A and B, and Fig. 3, A and B, did not describe the data better than the Hill equations in Fig. 1, B and D, indicating that this model is not appropriate to describe the interaction of PnIA and [A10L]PnIA with the receptor. The observation that brief (0.5 or 1 s) applications or low concentrations of PnIA and [A10L]PnIA increased the peak current amplitude above control values indicate that the scheme involving binding is complex, and this may affect the fit to the data.

**[A10L]PnIA Alanine Mutants**—To investigate the contribution of amino acid side chains in the [A10L]PnIA molecule to the inhibitory potency at  $\alpha 7$  receptors, we examined a series of alanine mutants of [A10L]PnIA. All alanine mutants of [A10L]PnIA significantly inhibited currents at wt $\alpha 7$  receptors ( $p < 0.05$ ). Fig. 4 shows a comparison of the inhibitory effects of the [A10L]PnIA alanine mutants (200 nM) on chick  $\alpha 7$  nAChRs. Removal of the projecting side chains between position 5 and 13 and at position 15, by the insertion of an alanine residue, significantly reduced the potency of 200 nM [A10L]PnIA to inhibit ACh-evoked currents ( $p < 0.05$ ).

**PnIA and [A10L]PnIA Have Different Effects on  $\alpha 7$ -L247T Receptors**—The ability of PnIA and [A10L]PnIA to inhibit cur-

<sup>2</sup> R. C. Hogg, G. Hopping, P. F. Alewood, D. J. Adams, and D. Bertrand, unpublished results.

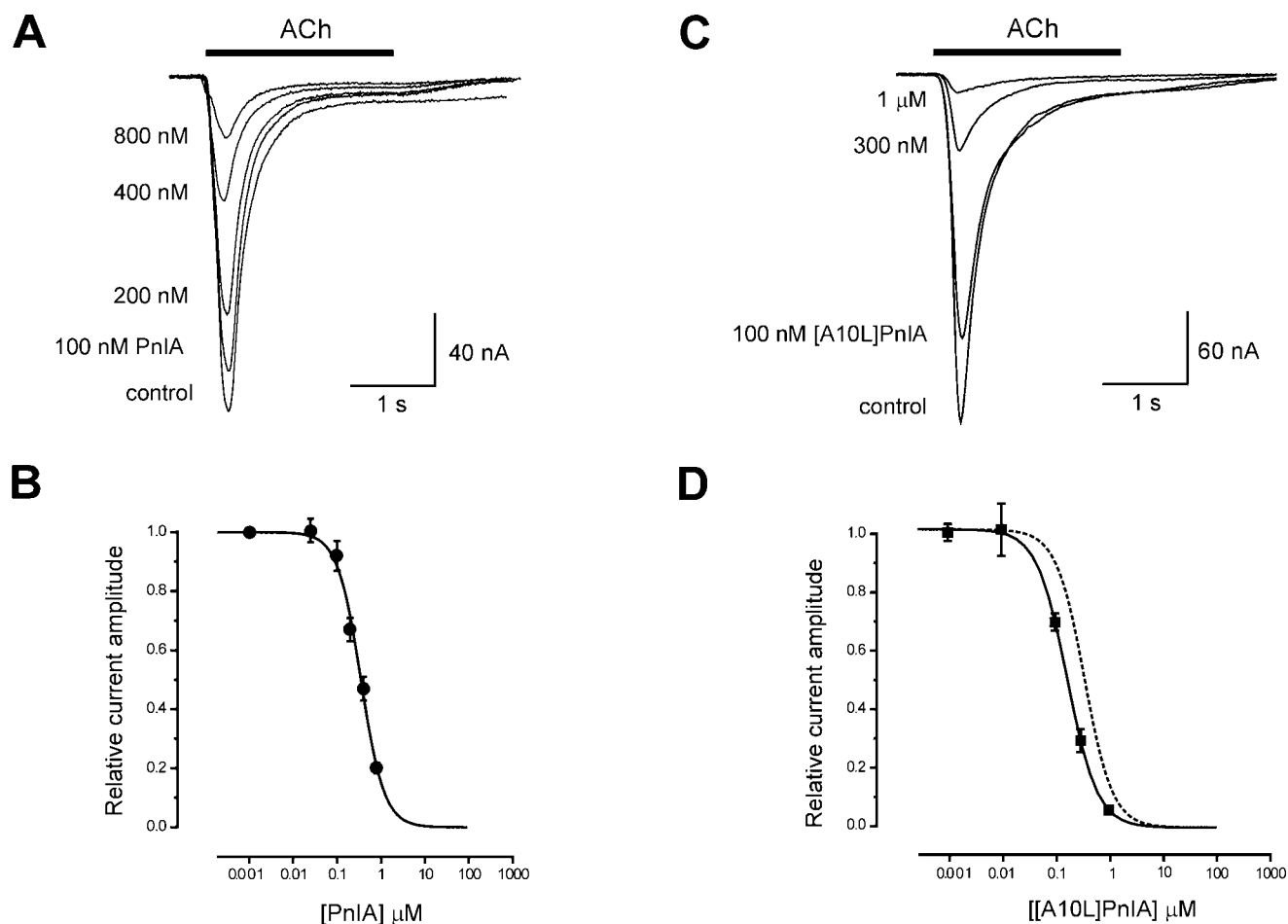


FIG. 1. Effect of PnIA and [A10L]PnIA on ACh-activated currents in wt $\alpha 7$  receptors. *A*, PnIA inhibits ACh-activated currents in wt $\alpha 7$  receptors in a concentration-dependent manner. *B*, the curve fit to the data had an  $IC_{50}$  of 349 nM and a Hill coefficient of 1.8. *C*, [A10L]PnIA inhibits ACh-activated currents with an  $IC_{50}$  of 168 nM; the inhibition curve for PnIA has been superimposed for comparison. *D* (dashed line), Hill coefficient was 1.55. Currents were activated with 200  $\mu M$  ACh.

rents mediated by the wt $\alpha 7$  receptor indicates that they stabilize a state of the receptor which is non-conducting. Since the A10L mutation changes the selectivity of the toxin for receptor subtypes (7, 8), it is possible that the mechanism of inhibition may also be different. In the minimal hypothetical gating scheme for a nAChR shown in Fig. 5, this could be either the resting or the desensitized state (*D*), which are both non-conducting in the wt $\alpha 7$  receptor.

To investigate whether PnIA and [A10L]PnIA stabilize the same state of the receptor, we used the chick  $\alpha 7$  receptor containing the L247T mutation.  $\alpha 7$ -L247T receptors display non-desensitizing currents in response to ACh, and it has been proposed that the L247T mutation causes one of the desensitized states of the receptor to become conducting (12); this desensitized open state is represented as  $D^*$  in Fig. 5 and could account for the non-desensitizing current. It has been proposed that it is this state of the receptor that is stabilized by nAChR antagonists, such as DH $\beta$ E, which activate currents at this receptor (14). Occasionally, slow desensitization of  $\alpha 7$ -L247T receptors is observed, making it likely that other desensitized non-conducting states of the receptor also exist. The  $\alpha 7$ -L247T receptors exhibited a slowly activating, non-desensitizing current in response to ACh which reached a plateau within  $\sim 10$  s.  $\alpha 7$ -L247T receptors show an increased sensitivity to ACh compared with wild type receptors with an  $EC_{50}$  of 0.62  $\mu M$  (14, 19). PnIA inhibited the ACh-induced current through  $\alpha 7$ -L247T receptors in a concentration-dependent and reversible manner (Fig. 6*A*). The dose-response relationship was fit by a curve with an  $IC_{50}$  of 194 nM and a slope of 0.9 (Fig. 6*B*).

A proportion of the  $\alpha 7$ -L247T receptors are spontaneously active (14). PnIA also inhibited the spontaneously active “leak” current  $\alpha 7$ -L247T receptors in a concentration-dependent manner (data not shown). In contrast to the inhibitory effect of PnIA at  $\alpha 7$ -L247T receptors, co-application of [A10L]PnIA further activated the ACh-evoked current (Fig. 6*C*), suggesting that [A10L]PnIA and the alanine mutants stabilize a conducting state of the receptor. All the alanine mutants of [A10L]PnIA had a similar activating effect on the ACh-evoked currents at the  $\alpha 7$ -L247T receptors (see Fig. 6*C* and Table II). [A10L]PnIA (200 nM) increased the ACh-activated current to  $1.79 \pm 0.09$  times control ( $n = 9$ ). The mean amplitude of the [A10L]PnIA-activated current was  $307 \pm 196$  nA ( $n = 9$ ). [A10L]PnIA was also able to evoke a current at  $\alpha 7$ -L247T receptors in the absence of ACh (Fig. 6*D*), further suggesting that [A10L]PnIA stabilizes an “open state” of the receptor.

#### DISCUSSION

The aim of this study was to investigate the molecular interactions between  $\alpha$ -conotoxins and the  $\alpha 7$  nAChR and to determine what effect these have on toxin activity. To this end, we have used a series of mutated toxins and compared their effects on wild type and mutant nAChRs to determine whether mutation of the native toxin changes the mechanism of inhibition, in particular the affinity for different states of the receptor. We have examined which amino acid side chains might be responsible for determining specificity of binding to different receptor states.

PnIA and [A10L]PnIA both inhibit ACh-evoked currents at wt $\alpha 7$  receptors. [A10L]PnIA was approximately twice as potent

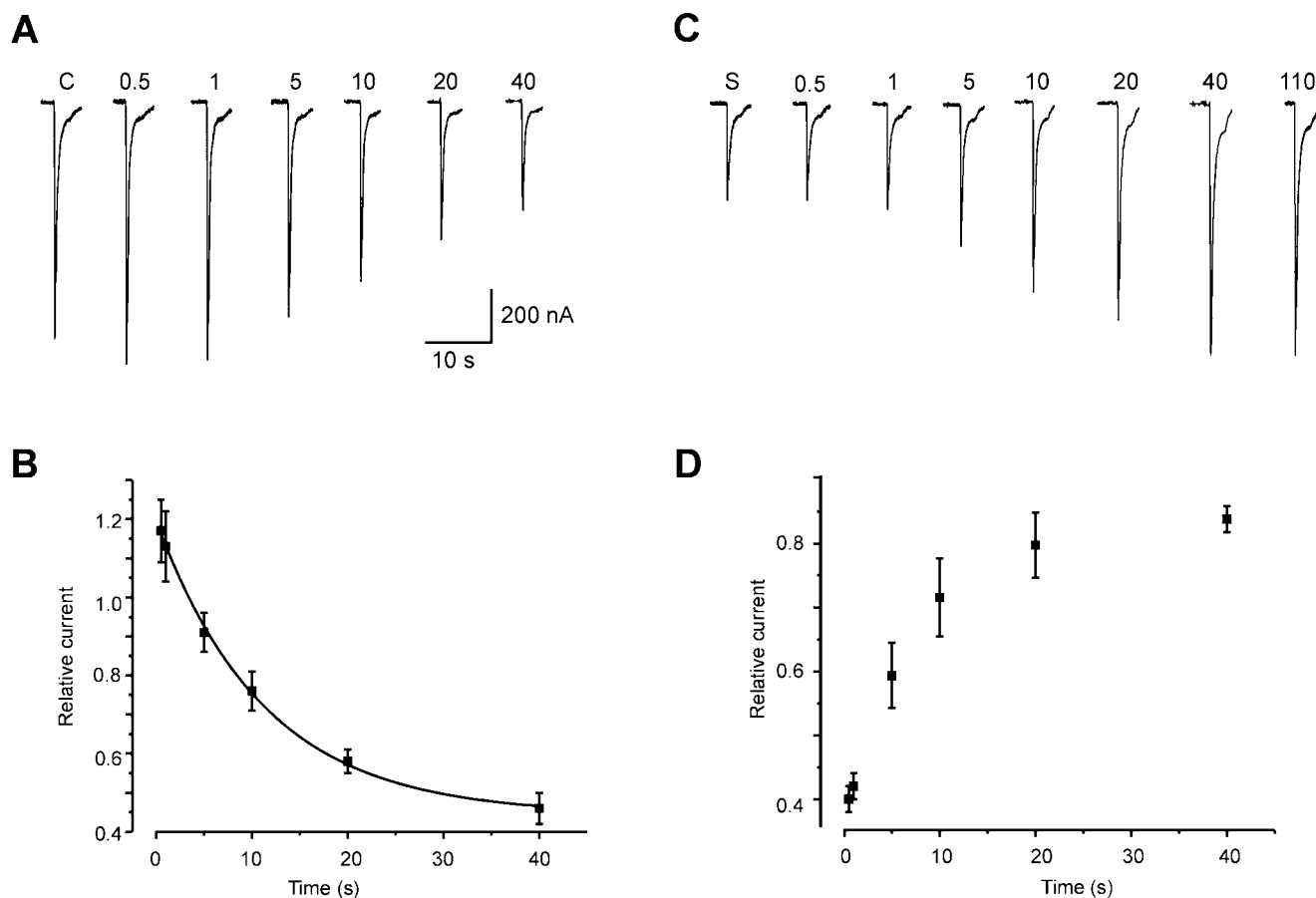


FIG. 2. Time course of block and recovery from block of PnIA at wt $\alpha 7$  receptors. A, 400 nM PnIA progressively inhibited ACh-induced responses (200  $\mu$ M). B, the time course of onset of block was fit by a single exponential with a  $\tau$  of 11 s. C, recovery of ACh-induced current amplitude upon washout of PnIA. D, time course of recovery of current amplitude from block by 400 nM PnIA.  $n = 5$  for each data point.

as PnIA. The rates of onset of block were similar for both toxins, whereas recovery from block was slower for [A10L]PnIA. These data suggest that the greater potency of [A10L]PnIA at  $\alpha 7$  receptors is conveyed by a slower rate of dissociation from the receptor. The rate of bath exchange was  $< 1$  s, which is rapid compared with the onset of inhibition, making it unlikely that the rate of toxin binding is diffusion limited. A similar difference in off rates of PnIA and [A10L]PnIA has been observed at cloned rat  $\alpha 7$  receptors (8). Attempts to describe toxin blockade using the model proposed for MLA inhibition of  $\alpha 7$  receptors revealed that marked differences in the profile of recovery as well as steady-state inhibition exist between  $\alpha$ -conotoxin inhibition and MLA. The absence of a lag phase at the onset of the recovery can be attributed to either reflecting profound differences in the mechanism of inhibition or as an inability to resolve the recovery phase with adequate time resolution. Interestingly, Luo *et al.* (8) have previously shown that both the onset and recovery of PnIA and [A10L]PnIA blockade can be fitted with a single exponential. However, the Hill coefficient greater than unity observed for the steady-state dose-response inhibition indicates that the toxin inhibition cannot be described by a simple bimolecular reaction.

NMR analysis has shown that the A10L substitution in PnIA does not affect the backbone structure of the molecule or the angle of the projecting side chains (7); therefore, this increase in potency must be attributed to the longer aliphatic side chain of the leucine at position 10. The circular dichroism spectra of the [A10L]PnIA mutants were unchanged with regard to the native toxin, indicating that the  $\alpha$ -helix is still present.<sup>2</sup>

All alanine mutants of [A10L]PnIA inhibited ACh-evoked

currents at wt $\alpha 7$  receptors. The potency profile of these toxins indicates that removal of the projecting amino acid side chains between position 5 and 13 and at position 15, by the insertion of an alanine residue, reduced the potency of the [A10L]PnIA mutants to inhibit ACh-evoked currents at wt $\alpha 7$  receptors. The pairwise interactions involved in the binding of PnIA and PnIB to the human  $\alpha 7/5$ HT-3 receptor has been investigated by mutant cycle analysis combined with a competitive binding assay (21). The sequences of PnIB and [A10L]PnIA are similar, differing only by a single serine in place of the asparagine residue at position 11. The residues at positions 4, 5, 6, 7, 9, and 10 were found to endow PnIB with an affinity for the  $\alpha 7/5$ HT-3 receptor. This study reported a dominant interaction between the leucine at position 10 and Trp-149 in loop B of the positive face of the ACh binding site, which anchors the toxin to the receptor, and also interactions between the proline residues at positions 6 and 7 of the toxin with the receptor. Data from the present study indicates that side chains projecting from the helical central portion of the molecule contribute to the functional blocking activity of [A10L]PnIA at  $\alpha 7$  receptors. Since the previous study measured toxin binding in the absence of agonist (21) and the present study functional activity, it is possible that the toxin may be binding to different resting or desensitized states of the receptor. The modification of the projecting side chains may change the affinity of the toxin to stabilize the receptor in alternative closed states.

The presence of a longer aliphatic side chain at position 10 in PnIA has been reported to increase the affinity of the toxin for  $\alpha 7$  receptors (21). Functional studies have shown that A10L mutation in PnIA makes the resulting [A10L]PnIA toxin more selective for  $\alpha 7$  receptors (7, 8). To determine whether this

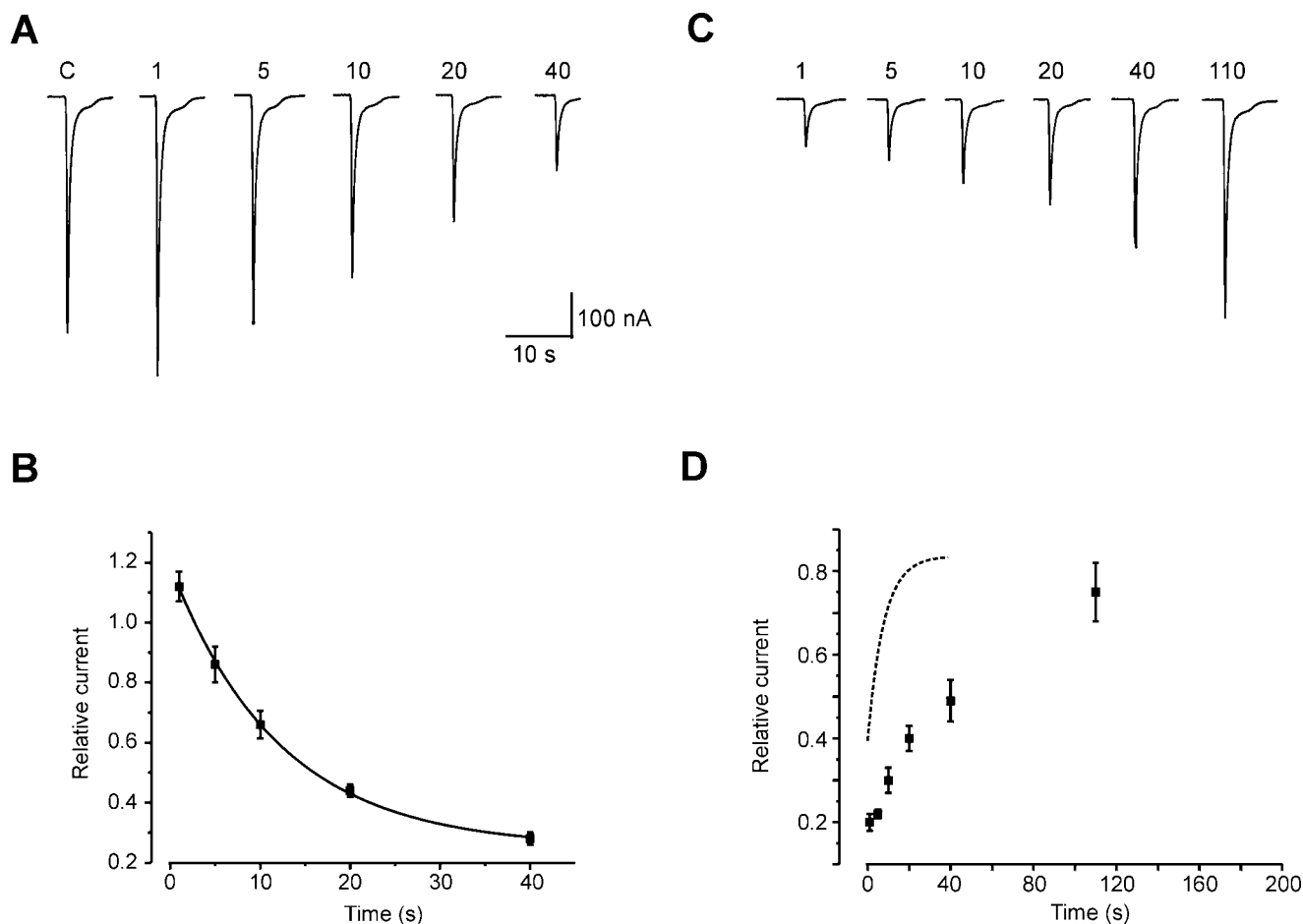


FIG. 3. **Time course of block and recovery from block of [A10L]PnIA at wt $\alpha$ 7 receptors.** A, 200 nM [A10L]PnIA inhibited responses to ACh (200  $\mu$ M). B, data points for the time course of onset of block were fit by single exponential curve with a  $\tau$  value of 12 s. C, recovery of ACh-induced current amplitude upon washout of [A10L]PnIA. D, time course of recovery from block was significantly slower with a half-recovery time in the region of 70 s,  $n = 4$  for each data point. Dashed line represents the recovery from block with PnIA.

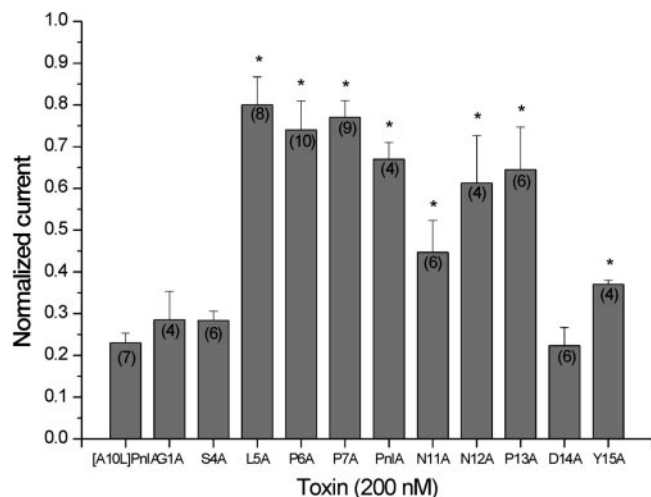


FIG. 4. **Relative inhibition of ACh-induced currents at wt $\alpha$ 7 receptors by PnIA, [A10L]PnIA, and alanine analogues.** Control current amplitudes in the absence of toxin have been normalized to 1.0. All toxins were applied at 200 nM, and currents were activated by 200  $\mu$ M ACh. The number of experiments is given in brackets, and asterisks indicates values significantly different from [A10L]PnIA ( $p < 0.05$ ).

mutation also changes the affinity of the toxin for different states of the  $\alpha$ 7 receptor, we used the  $\alpha$ 7-L247T mutant receptor. Two models have been proposed to account for the non-desensitizing behavior of the L247T mutant. One is that the desensitized state of the receptor becomes conducting, and

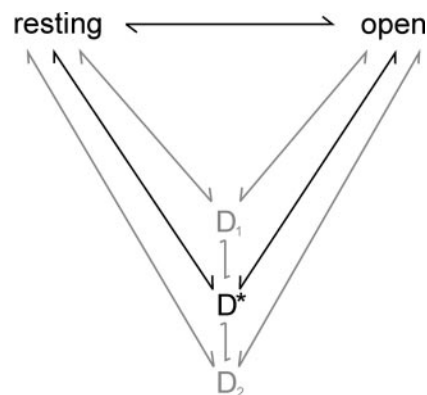
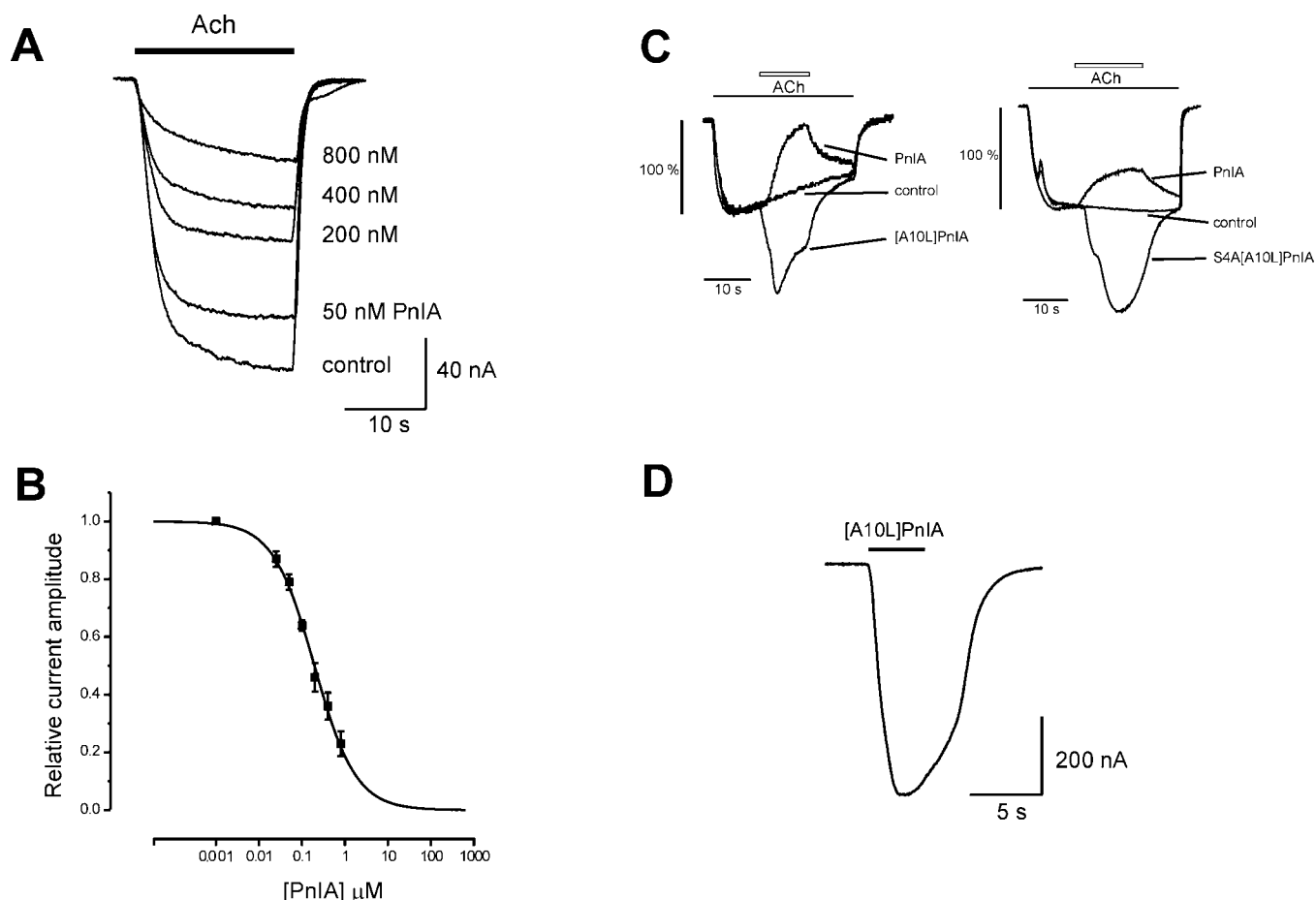


FIG. 5. **Hypothetical gating scheme for the nAChR.** The nAChR is assumed to exist in multiple interconvertible states, a closed resting state, an open conducting state, and at least one desensitized state. In wild type receptors the desensitized states are non-conducting; however, we propose that in the  $\alpha$ 7-L247T receptor one of the desensitized states ( $D^*$ ) of the receptor is conducting.

another proposes that the isomerization coefficient is altered favoring the open state (22–24). However, this second scheme does not adequately describe how antagonists of the wt $\alpha$ 7 receptor can act as agonists of the mutant receptor. If we assume the first model, in which the toxin is stabilizing the desensitized state of the receptor, we would expect to see a much reduced block at the  $\alpha$ 7-L247T receptor.

PnIA potentially inhibited ACh-induced currents at  $\alpha$ 7-L247T receptors, indicating that, as for MLA and  $\alpha$ -bungarotoxin, it



**FIG. 6. PnIA inhibits the ACh-evoked current in  $\alpha$ 7-L247T mutant receptors, whereas [A10L]PnIA and the alanine mutants facilitate ACh-induced responses.** *A*, PnIA inhibits non-desensitizing ACh-evoked currents in  $\alpha$ 7-L247T receptors. *B*, concentration-response relationship for inhibition had an  $IC_{50}$  of 194 nM, and ACh concentration was 0.5  $\mu$ M. *C*, PnIA, and [A10L]PnIA and alanine mutants, have different effects on the ACh-evoked current at  $\alpha$ 7-L247T receptors; PnIA (200 nM) inhibits, while [A10L]PnIA (200 nM) activates, currents in the same oocyte. S4A[A10L]PnIA also activates a current, while PnIA inhibits the ACh-evoked current. The *black bar* indicates the application of ACh (0.5  $\mu$ M), and the *open bar* indicates the toxin application. Currents have been normalized and superimposed, and control current amplitude was 250 nA. *D*, [A10L]PnIA (1  $\mu$ M) activates a current at  $\alpha$ 7-L247T receptors.

**TABLE II**  
Effect of  $\alpha$ -conotoxins on ACh-activated currents at  $\alpha$ 7-L247T receptors

Toxin	Activation	Inhibition
[A10L]PnIA	+	
G1A	+	
S4A	+	
L5A	+	
P6A	+	
P7A	+	
PnIA		+
N11A	+	
N12A	+	
P13A	+	
D14A	+	
Y15A	+	

probably stabilizes the receptor in the resting state. However, in contrast, [A10L]PnIA and the [A10L]PnIA alanine scan mutants all potentiated the ACh-induced responses at the  $\alpha$ 7-L247T receptors. In addition, [A10L]PnIA was able to activate a current at  $\alpha$ 7-L247T receptors in the absence of ACh. Since [A10L]PnIA potentially inhibits currents at wt $\alpha$ 7 receptors but potentiates currents at the  $\alpha$ 7-L247T mutant, we conclude that [A10L]PnIA is stabilizing the desensitized state of the receptor that is non-conducting in the wt $\alpha$ 7 receptor but conducting in the  $\alpha$ 7-L247T mutant. PnIA stabilizes the receptor in a state that is non-conducting in both receptors, which would corre-

spond to the resting state. The ability of all the toxins containing the [A10L] mutation to potentiate the currents at  $\alpha$ 7-L247T receptors indicates the presence of the longer lysine side chain at position 10 alone is sufficient to change the specificity of the toxin to bind and stabilize the  $\alpha$ 7-L247T receptor in different states, and this is not affected by other mutations. The observation that the amplitude of the ACh-induced current at wt $\alpha$ 7 receptors was increased following short 0.5–1-s applications of PnIA and [A10L]PnIA (Figs. 2A and 3A) suggests both toxins bind to the resting state of the receptor at low concentrations and increase the ratio of receptors that are in the resting as compared with the desensitized state. This effect supports the hypothesis that PnIA is stabilizing the resting state of the receptor, but also suggests that at low concentrations [A10L]PnIA may also have some affinity for a resting state of the receptor. However, since the resting and desensitized states of the wt $\alpha$ 7 receptor are both non-conducting, there is no way to confirm that [A10L]PnIA stabilizes the same state in the wt $\alpha$ 7 and  $\alpha$ 7-L247T receptor.

Fig. 4 illustrates that the removal of side chains from residues between positions 5 and 13 reduce the potency of [A10L]PnIA to inhibit currents at  $\alpha$ 7 receptors. However, it is clear that the removal of projecting side chains at positions other than position 10 does not affect the ability of [A10L]PnIA to potentiate responses at  $\alpha$ 7-L247T receptors.

While demonstrating unambiguously the ability of [A10L]PnIA to stabilize the desensitized open state of the  $\alpha$ 7-

L247T receptor, the rather complex form of the evoked current indicates that a complex equilibrium must be stabilized by the toxin. Since it is beyond the scope of this study to examine these time courses in detail, no attempt was made to analyze the profile of current decay.

This study highlights the usefulness of the  $\alpha$ -conotoxins as research tools, which can be subtly modified to probe the molecular interactions of ligand binding to nAChRs. The stability of the peptide backbone to amino acid substitutions also makes them ideal models to develop pharmacophores for nAChR subtypes, which may lead to the development of non-peptide modulators of nAChR function.

## REFERENCES

- Adams, D. J., Alewood, P. F., Craik, D. J., Drinkwater, R., and Lewis, R. J. (1998) *Drug Dev. Res.* **46**, 219–234
- McIntosh, J. M., and Jones, R. M. (2001) *Toxicol.* **39**, 1447–1451
- McIntosh, J. M., Santos, A. D., and Olivera, B. M. (1999) *Annu. Rev. Biochem.* **68**, 59–88
- Arias, H. R., and Blanton, M. P. (2000) *Int. J. Biochem. Cell Biol.* **32**, 1017–1028
- Hu, S. H., Gehrman, J., Guddat, L. W., Alewood, P. F., Craik, D. J., and Martin, J. L. (1996) *Structure (Lond.)* **4**, 417–423
- Fainzilber, M., Hasson, A., Oren, R., Burlingame, A. L., Gordon, D., Spira, M. E., and Zlotkin, E. (1994) *Biochemistry* **33**, 9523–9529
- Hogg, R. C., Miranda, L. P., Craik, D. J., Lewis, R. J., Alewood, P. F., and Adams, D. J. (1999) *J. Biol. Chem.* **274**, 36559–36564
- Luo, S., Nguyen, T. A., Cartier, G. E., Olivera, B. M., Yoshikami, D., and McIntosh, J. M. (1999) *Biochemistry* **38**, 14542–14548
- Changeux, J. P. (1990) *Trends Pharmacol. Sci.* **11**, 485–492
- Monod, J., Wyman, J., and Changeux, J. P. (1965) *J. Mol. Biol.* **12**, 88–118
- Edelstein, S. J., Schaad, O., Henry, E., Bertrand, D., and Changeux, J. P. (1996) *Biol. Cybern.* **75**, 361–379
- Bertrand, D., Devillers-Thiery, A., Revah, F., Galzi, J. L., Hussy, N., Mulle, C., Bertrand, S., Ballivet, M., and Changeux, J. P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 1261–1265
- Bertrand, D., Galzi, J. L., Devillers-Thiery, A., Bertrand, S., and Changeux, J. P. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6971–6975
- Bertrand, S., Devillers-Thiery, A., Palma, E., Buisson, B., Edelstein, S. J., Corringer, P. J., Changeux, J. P., and Bertrand, D. (1997) *Neuroreport* **8**, 3591–3596
- Palma, E., Mileo, A. M., Eusebi, F., and Miledi, R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 11231–11235
- Palma, E., Maggi, L., Eusebi, F., and Miledi, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 9915–9919
- Bertrand, D., Cooper, E., Valera, S., Rungger, D., and Ballivet, M. (1991) *Methods Neurosci.* **4**, 174–193
- Schnolzer, M., Alewood, P., Jones, A., Alewood, D., and Kent, S. B. (1992) *Int. J. Pept. Protein Res.* **40**, 180–193
- Revah, F., Bertrand, D., Galzi, J. L., Devillers-Thiery, A., Mulle, C., Hussy, N., Bertrand, S., Ballivet, M., and Changeux, J. P. (1991) *Nature* **353**, 846–849
- Palma, E., Bertrand, S., Binzoni, T., and Bertrand, D. (1996) *J. Physiol. (Lond.)* **491**, 151–161
- Quiram, P. A., McIntosh, J. M., and Sine, S. M. (2000) *J. Biol. Chem.* **275**, 4889–4896
- Filatov, G. N., and White, M. M. (1995) *Mol. Pharmacol.* **48**, 379–384
- Labarca, C., Nowak, M. W., Zhang, H., Tang, L., Deshpande, P., and Lester, H. A. (1995) *Nature* **376**, 514–516
- Palma, E., Eusebi, F., and Miledi, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 1539–1543

**$\alpha$ -Conotoxins PnIA and [A10L]PnIA Stabilize Different States of the  $\alpha$ 7-L247T  
Nicotinic Acetylcholine Receptor**

Ron C. Hogg, Gene Hopping, Paul F. Alewood, David J. Adams and Daniel Bertrand

*J. Biol. Chem.* 2003, 278:26908-26914.

doi: 10.1074/jbc.M212628200 originally published online May 13, 2003

---

Access the most updated version of this article at doi: [10.1074/jbc.M212628200](https://doi.org/10.1074/jbc.M212628200)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 24 references, 8 of which can be accessed free at  
<http://www.jbc.org/content/278/29/26908.full.html#ref-list-1>