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Membrane Transport, Structure, Function, and Biogenesis: 
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β2 Subunit Contribution to 4/7 α-Conotoxin Binding to the Nicotinic Acetylcholine Receptor*

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The structures of acetylcholine-binding protein (AChBP) and nicotinic acetylcholine receptor (nAChR) homology models have been used to interpret data from mutagenesis experiments at the nAChR. However, little is known about AChBP-derived structures as predictive tools. Molecular surface analysis of nAChR models has revealed a conserved cleft as the likely binding site for the 4/7 α-conotoxins. Here, we used an α3β2 model to identify β2 subunit residues in this cleft and investigated their influence on the binding of α-conotoxins MII, PnIA, and GID to the α3β2 nAChR by two-electrode voltage clamp analysis. Although a β2-L119Q mutation strongly reduced the affinity of all three α-conotoxins, β2-F117A, β2-V109A, and β2-V109G mutations selectively enhanced the binding of MII and GID. An increased activity of α-conotoxins GID and MII was also observed when the β2-F117A mutant was combined with the α4 instead of the α3 subunit. Investigation of A10L-PnIA indicated that high affinity binding to β2-F117A, β2-V109A, and β2-V109G mutants was conferred by amino acids with a long side chain in position 10 (PnIA numbering). Docking simulations of 4/7 α-conotoxin binding to the α3β2 model supported a direct interaction between mutated nAChR residues and α-conotoxin residues 6, 7, and 10. Taken together, these data provide evidence that the β subunit contributes to α-conotoxin binding and selectivity and demonstrate that a small cleft leading to the agonist binding site is targeted by α-conotoxins to block the nAChR.

The neuronal nicotinic acetylcholine receptors (nAChRs) comprise a large family of ion channels formed by the heteropentamic assembly of homologous subunits. α-Conotoxins, small disulfide-rich peptides isolated from the venom of predatory cone snails, potently and selectively block nAChRs (1). They act as competitive antagonists at the ACh binding site, which is interrupted by a cleft above the N-terminal half of the 4/7 α-conotoxins structure and the cleft. Upon examination of this model, it was apparent that the β2 subunit contributed more than the α subunit to the formation of this cleft, suggesting an important role of β-residues for binding and/or selectivity. In this study, we mutated three residues in the β2 subunit residues located in this cleft to characterize the contribution of the β subunit to α-conotoxin binding. Each of these mutants influenced the binding of α-conotoxins MII, GID, and PnIA. Additionally, molecular surface analysis revealed striking shape complementarity between the highly conserved N-terminal half of the 4/7 α-conotoxins structure and the cleft. Docking experiments showed that both MII and PnIA bind deep into the α3β2 nAChR cleft, explaining at the molecular level the experimental results obtained from mutagenesis studies. Based on these results, we propose that the conserved cleft above the β9β10 hairpin is the binding site for 4/7 α-conotoxins active at α7, α3β2, and α4β2 nAChRs and that the α-conotoxins subtypes and even between different binding sites within the same receptor. As a result, they are widely used as pharmacological tools for the subtype differentiation of nAChRs in native tissues (2).
EXPERIMENTAL PROCEDURES

Materials—cDNAs encoding neuronal nAChRs were provided by J. Patrick (Baylor College of Medicine, Houston, TX) and subcloned into the oocyte expression vector pNKS2 (18). MII, PnIA, and GID were gifts from J. T. Blanchfield (School of Pharmacy, The University of Queensland, Australia), G. Hopping and M. Loughnan (Institute for Molecular Bioscience, The University of Queensland, Australia), respectively, and were synthesized as described previously (9, 19, 20).

Homology Modeling—The FASTA format of the \( \alpha_3 \beta_2 \) rat sequences were retrieved from the ligand-gated ion channel data base (pasteur.fr/recherche/banques/LGIC/). Their extracellular ligand-binding domains were aligned with the AChBP sequence as described previously (21).

A homology model of the \( \alpha_3 \beta_2 \) nAChR subtype was built on a Silicon Graphics Octane R12000 work station using the MODELLER program (22). The AChBP structure (1I9B) was loaded in the INSIGHT II (Accelrys, San Diego, CA) environment and used as a template. Three models were built with a high “optimize level.” The two options “optimize loop models” and “loop optimize level” were set to 3 and high, respectively. User disulfide was selected to assign the disulfide bonds as in AChBP except for the missing vicinal disulfide in the \( \beta_2 \) subunit. The model with the lowest root mean square deviation compared with AChBP was refined further and used for these studies. Steepest descent energy minimizations were applied using the AMBER force field and DISCOVER program implemented in Insight II.

Mutagenesis—Mutagenesis of the \( \beta_2 \) nAChR subunit cDNA was achieved using the QuikChange™ site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. Primers used to generate the mutants were from Proligo (Lismore, Australia). All mutations were confirmed by cDNA sequencing.

Electrophysiological Recordings—cRNA was synthesized from linearized plasmids with SP6 RNA polymerase using the mMessageMachine kit (Ambion, Austin, TX). Xenopus laevis frogs were purchased from Nasco International (Fort Atkinson, WI) or Firma Kahler (Hamburg, Germany). X. laevis oocytes were prepared as described previously (6) and injected with 50-nl aliquots of cRNA (0.5 μg/μl).

Fig. 1. Homology model of the \( \alpha_3 \beta_2 \) nAChR showing the location of residues mutated in this study. A, ribbon representation of \( \alpha_3 \beta_2 \) nAChR homology model with mutated residues in stick representation. B, enlarged view of the molecular surface of the \( \alpha_3 \beta_2 \) subunit interface showing the relative contribution of both \( \alpha_3 \) (purple) and \( \beta_2 \) (white) subunits to the cleft. Mutated residues are indicated.

Fig. 2. Concentration-response analysis of \( \alpha \)-conotoxin MII (A), GID (B), PnIA (C), and \([\alpha_{10L}]\)-PnIA (D) on wild type and mutant nAChRs. The indicated subunit combinations were expressed in Xenopus oocytes and analyzed by 2-electrode voltage clamp. Responses to 2-s pulses of 100 μM ACh were recorded after a 3-min preincubation with the indicated toxin. IC\(_{50}\) values and Hill slopes are given in Table I. Each point represents the average of at least three measurements. Error bars represent S.E.

Two-electrode voltage clamp recordings were performed in oocytes 1–10 days after cRNA injection at a holding potential of −70 mV. Pipettes were pulled from borosilicate glass and filled with 3 M KCl. Resistances were below 1 megohm. Membrane currents were recorded using a Turbo Tec-10CX or a Turbo Tec 65X Amplifier (npi electronic, Tamm, Germany) filtered at 200 Hz and digitized at 400 Hz. Version
8.53 Pulse software (HEKA Elektronik, Lambrecht, Germany) or Cell-works software were used. The perfusion medium was automatically switched between ND96 with or without agonist (100 µM ACh) using a custom-made magnetic valve system. A fast and reproducible solution exchange (<300 ms) for agonist application was achieved using a 50-µl funnel-shaped oocyte chamber combined with a fast solution flow (~150 µl/s) fed through a custom-made manifold mounted immediately above the oocyte. ACh pulses were applied for 2 s at 4-min intervals. After each application, the cell was superfused for 1 min with agonist-free solution, and the flow was then stopped for 3 min. Peptide was mixed from a 10-fold stock into the static bath when responses to three consecutive agonist applications differed by less than 10%. Addition of toxin directly to the recording chamber preserved material and avoided adhesion of the toxin to tubing surfaces. To obtain estimates of potency, dose–response curves were fit to the data by the equation % response = 100/[1 + ((toxin)/IC_{50})^n] using Prism software (GraphPad version 3.0 for Macintosh, San Diego, CA). To obtain estimates of toxin dissociation rates, agonist responses were measured at 2-min intervals under constant superfusion, and after stabilization of responses, oocytes were incubated with toxin in a static bath for 1 min. To obtain estimates of the toxin association rate, oocytes were continuously superfused with solution containing the indicated toxin concentration and ACh pulses were applied in 1- or 2-min intervals. Association and dissociation curves were fit to the data by the equations % response = span (1 - e^{-K \times t_{\text{time}}}), and plateau and % response = span (1 - e^{-K \times t_{\text{time}}}) plateau, respectively.

Docking Experiments—Docking simulations of α-conotoxins were carried out as described in Dutertre et al. (17). Briefly, MII and PnIA structures were retrieved from the protein data bank (Protein Data Bank codes 1MII and 1PEN, respectively) and docked onto an α3β2 homology model using the program GOLD, version 1.2 (Genetic Optimization for Ligand Docking, Cambridge Crystallographic Data Centre, Cambridge, UK). One NMR/crystal structure was chosen for each α-conotoxin, as GOLD treats ligands as flexible molecules with side chain orientations optimized during calculations. As conotoxins are competitive antagonists, the active site radius was set at 20 Å from

### Table I

| α-Conotoxins | IC_{50} values and 95% confidence interval for α-conotoxins on wild type and mutant nAChR combinations |
|--------------|------------------------------------------------------------------------------------------------------------------|
| MII          |                                                                                                                  |
| a3β2         | 6.08 (4.87–7.61)                                               | 1.38 (0.94–1.83)                                                |
| a3β2F117A    | 4.66 (3.59–6.05)                                               | 1.04 (0.80–1.27)                                                |
| a3β2V109A    | 3.98 (3.58–4.43)                                               | 1.22 (1.10–1.33)                                                |
| a3β2V109G    | 3.51 (3.70–5.75)                                               | 1.29 (1.01–1.56)                                                |
| a3β2L119Q    | 1,810 (1,380–2,380)                                            | 0.99 (0.73–1.25)                                                |
| GID          |                                                                                                                  |
| a3β2         | 5.09 (4.17–6.22)                                               | 1.60 (1.11–2.10)                                                |
| a3β2F117A    | 3.52 (2.81–4.42)                                               | 1.29 (0.90–1.68)                                                |
| a3β2V109A    | 4.91 (4.39–5.48)                                               | 1.52 (1.26–1.78)                                                |
| a3β2V109G    | 3.72 (3.27–4.22)                                               | 1.45 (1.18–1.71)                                                |
| a3β2L119Q    | 839.20 (752.8–935.5)                                           | 0.90 (0.79–1.01)                                                |
| PnA          |                                                                                                                  |
| a3β2         | 7.74 (6.79–8.81)                                               | 0.85 (0.74–0.97)                                                |
| a3β2F117A    | 7.81 (6.71–9.08)                                               | 0.93 (0.79–1.07)                                                |
| a3β2V109A    | 10.12 (7.11–14.43)                                             | 1.06 (0.64–1.47)                                                |
| a3β2V109G    | 34.39 (26.47–44.68)                                            | 0.86 (0.60–1.12)                                                |
| a3β2L119Q    | 295.80 (177.3–493.6)                                           | 0.88 (0.54–1.23)                                                |

### Table II

| α-Conotoxins | k_{off} \text{ min}^{-1} |
|--------------|--------------------------|
| MII          |                           |
| a3β2         | 0.07 (0.04–0.1)           |
| a3β2F117A    | ND* (8.9 ± 0.7% recovery in 20 min) |
| a3β2V109A    | ND (11.4 ± 2.6% recovery in 20 min) |
| a3β2V109G    | ND (14.1 ± 2.5% recovery in 20 min) |
| GID          |                           |
| a3β2         | 0.19 (0.15–0.22)          |
| a3β2F117A    | ND (15.2 ± 3.0% recovery in 20 min) |
| a3β2V109A    | 0.09 (0.06–0.12)          |
| a3β2V109G    | 0.03 (0.01–0.05)          |
| PnA          |                           |
| a3β2         | ND (100% recovery in 2 min) |
| a3β2F117A    | ND (100% recovery in 4 min) |
| a3β2V109A    | ND (100% recovery in 4 min) |
| a3β2V109G    | ND (100% recovery in 2 min) |
| [A10L]PnA    |                           |
| a3β2         | ND (100% recovery in 2 min) |
| a3β2F117A    | 0.09 (0.07–0.11)          |
| a3β2V109A    | 0.12 (0.09–0.15)          |
| a3β2V109G    | 0.13 (0.12–0.33)          |

* ND, not determined.  
** 95% confidence interval values are shown in parentheses.

8.53 Pulse software (HEKA Elektronik, Lambrecht, Germany) or Cell-Works software were used. The perfusion medium was automatically switched between ND96 with or without agonist (100 µM ACh) using a custom-made magnetic valve system. A fast and reproducible solution exchange (<300 ms) for agonist application was achieved using a 50-µl funnel-shaped oocyte chamber combined with a fast solution flow (~150 µl/s) fed through a custom-made manifold mounted immediately above the oocyte. ACh pulses were applied for 2 s at 4-min intervals. After each application, the cell was superfused for 1 min with agonist-free solution, and the flow was then stopped for 3 min. Peptide was mixed from a 10-fold stock into the static bath when responses to three consecutive agonist applications differed by less than 10%. Addition of toxin directly to the recording chamber preserved material and avoided adhesion of the toxin to tubing surfaces. To obtain estimates of potency, dose–response curves were fit to the data by the equation % response = 100/[1 + ((toxin)/IC_{50})^n] using Prism software (GraphPad version 3.0 for Macintosh, San Diego, CA). To obtain estimates of toxin dissociation rates, agonist responses were measured at 2-min intervals under constant superfusion, and after stabilization of responses, oocytes were incubated with toxin in a static bath for 1 min. To obtain estimates of the toxin association rate, oocytes were continuously superfused with solution containing the indicated toxin concentration and ACh pulses were applied in 1- or 2-min intervals. Association and dissociation curves were fit to the data by the equations % response = span (1 - e^{-K \times t_{\text{time}}}), and plateau and % response = span (1 - e^{-K \times t_{\text{time}}}) plateau, respectively.
Trp-147 to ensure the analysis focused on residues around the ACh binding site. From the 100 docked structures obtained for each conotoxin, the selection of the final docked structure was based on a low scoring function determined in GOLD (23).

RESULTS AND DISCUSSION

Homology Modeling Identifies Val-109, Phe-117, and Leu-119 in the β2 nAChR Subunit as Likely Residues Interacting with α-Conotoxins MII and PnIA—We previously identified two cavities at opposite sides of the 9/10 hairpin (C-loop of an α2 subunit) on the surface of the nAChR, one large and easily accessible and one small and narrow, from which the ACh binding site could be reached (21). To differentiate between these two cavities and avoid confusion, we propose the use of “cavity” to refer to the larger one (below the 9/10 hairpin, close to the cell membrane), and “cleft” to refer to the smaller one (above the 9/10 hairpin). The cavity has recently been confirmed to represent the binding site used by the large snake neurotoxins to block the nAChR (24), whereas the cleft represents the likely region targeted by α-conotoxins (17, 21). Docking simulations with ImI and PnIB structures on an α3 nAChR model revealed overlapping but different binding sites for α-conotoxins and snake toxins (17). Both α-conotoxins block the receptor by targeting a small cleft above the 9/10 hairpin that is also blocked by the small peptide antagonist toxin waglerin (25) and the non-peptidic antagonist d-tubocurarine (26). Concerning the α3β2 nAChR, only one residue (β2-Thr-57) weakly affecting 4/7 conotoxin binding has been identified on the β2 subunit compared with four on the α3 subunit (27, 28). Interestingly, β2-Thr-57 is located above the 9/10 hairpin, forming part of the cleft in α3β2 together with three other residues, Val-109, Phe-117, and Leu-119. A potentially important role for additional residues in the β2 subunit that contribute to the binding and selectivity of 4/7 α-conotoxins, we mu.
Residue Leu-119 is Generally Important for α-Conotoxin Binding—A leucine in position β2-119 lies at the bottom of the cleft and is conserved in neuronal β4 and α7 nAChR subunits as well as in the muscle δ, γ, and ε nAChR subunits. Thus, a leucine is present at this location in all non-α subunits (and the (−) face of the α7 nAChR) for which highly active α-conotoxins have been identified. Indeed, no potent α-conotoxin has been found for the α9 and α10 subunits, which have a negatively charged aspartic acid at this position. In combination with the docking studies, this suggests that the hydrophobic environment created by β2-Leu-119 and the corresponding leucine residues in other subunits plays an important role in the high affinity interaction with α-conotoxins. To test the hypothesis that Leu-119 is generally important for α-conotoxin binding, the activity of three different α-conotoxins that bind with high affinity to the α3β2 nAChRs was tested on the hydrophilic α3β2-L119Q mutant. The α3β2/α6β2-selective α-conotoxin MII as well as the α3β2/α7-selective α-conotoxins PnIA and GID had a strongly reduced activity (Fig. 2, A–C), with 40-fold (PnIA), 165-fold (GID), and 300-fold (MII) higher IC50 values at this mutant α3β2 receptor (Table 1). This is the strongest effect described thus far for a non-α subunit mutant affecting α-conotoxin binding. Based on its position at the bottom of the cleft and the fact that the activity of all three conotoxins was reduced by at least 40-fold upon introduction of a similar length hydrophilic residue (Gln), Leu-119 may play an important stabilizing role for the interaction with α-conotoxins, allowing them to bind deep into the nAChR cleft.

In addition to the four cysteines, which are involved in two disulfide bonds and are mostly buried within the peptide core, only serine and proline residues are common to all three α-conotoxins. Because mutation of Ser-4 in MII did not affect potency (31), we suspect that Leu-119 might interact with the α-conotoxin backbone or the conserved proline found in 4/7 α-conotoxins (third residue of loop 1). However, this possibility is difficult to test by double mutant cycle analysis because alteration of the conserved cysteine or proline residues in the conotoxin alters the peptide backbone conformation.

Alanine Exchange of Val-109 and Phe-17 Specifically Enhances Binding of MII and GID—In our model of the β2 subunit, residues Val-109 and Phe-17 lie adjacent to each other on two neighboring antiparallel β-strands and above the Leu-119 residue, potentially forming the walls of the α-conotoxin binding cleft (Fig. 1). By mutating the residues into the smaller alanine, we expected to weaken the interaction with α-conotoxins. Unexpectedly, both the F117A and V109A mutations produced IC50 values for MII, GID, and PnIA little different from those at the wild type receptor, suggesting that Phe-117 and Val-109 do not have close interactions with the α-conotoxins.
The off-rates of MII were affected by all three except for PnIA, where a slight decrease in affinity was observed (Table II). Unfortunately, the long incubation times necessary to determine the real IC₅₀ values at the α3β2F117A and Val-109 mutants were not practical because of run-up/down effects on nAChR current seen in oocytes and the large amounts of toxin needed for superfusion. Therefore, to complement the IC₅₀ value determinations, we compared off-rates as a measure of toxin affinity that is independent of toxin concentration or preincubation time. Because not only the off-rate constants but also the on-rate constant of GID and MII on the Val-109 and Phe-117 mutants were decreased, we propose that these residues comprise part of a short access path or an intermediate binding state that α-conotoxins transition past before reaching (or leaving) their high affinity bound state.

The Effect on Val-109 and Phe-117 Exchange Also Occurs in Combination with the α4 nAChR Subunit—The results presented above show that a small modification of the β2 subunit can have a strong and specific effect on conotoxin binding, suggesting that the β subunit contributes to α-conotoxin binding through direct and specific interactions. However, it cannot be excluded that this is a secondary effect because of an alteration in the conformation of the α subunit induced by the mutation in the β subunit. To address this aspect, we investigated whether these β2 effects were preserved in combination with a different α subunit. Both MII and GID showed nanomolar activity at the α4β2 nAChR subtype, whereas no activity was observed for PnIA at concentration up to 1 μM. When the α4 subunit was combined with the β2-F117A mutation, both GID and MII showed an ~10-fold increase in activity (Fig. 5A) that was likewise paralleled by a clearly decreased off-rate (results not shown). This further indicates that the binding modes of MII and GID within the binding pocket are conserved across the α4β2 and α3β2 receptor interfaces and that the β subunit contributes directly to α-conotoxin potency. Whereas the overall higher IC₅₀ values at the α4β2 combination suggest that the contribution of the α4 subunit to conotoxin binding is smaller than that of the α3 subunit, the inactivity of PnIA could also be explained by a residue in PnIA that prevents an interaction with the α4 subunit.

The Length of the Side Chains in Position 10 of the α-Conotoxins Determines the Strength of the Interaction with the Val-109 and Phe-117 Mutants—Sequence comparison of PnIA with MII and GID (Fig. 6) shows that there are 7 residues in PnIA (Leu-5, Pro-7, Ala-7, Ala-10, Pro-13, Asp-14, and Tyr-15), which are different from the corresponding residues in GID or MII and thus are likely candidates to account for the poor activity of PnIA at the α4β2 interface and the low affinity of PnIA (compared with GID and MII) at the α3β2 mutants. Because MII has a leucine and PnIA an alanine at position 10, it appears that a long or bulky aliphatic side chain enhances α-conotoxin

![FIG. 7. Molecular surface complementarities for α-conotoxins and the α3β2 cleft.](Image)

![FIG. 8. Docking of PnIA (A) and MII α-conotoxins (B) onto the α3β2 nAChR.](Image)
interactions with the Phe-117 and Val-109 mutants. To further test this hypothesis, we investigated the activity of the A10L-PnIA analogue on both \(\alpha_9\beta_2\) and \(\alpha_9\beta_2\) mutants. As predicted, replacing Ala-10 by a longer leucine residue enhanced PnIA affinity for \(\alpha_9\beta_2\) receptors containing the Phe-117 or Val-109 mutations (Figs. 2D and 3D; Tables I and II). Indeed, a markedly reduced off-rate constant and an up to 23-fold lower IC\(_{50}\) value were observed for A10L-PnIA at the \(\alpha_9\beta_2\) mutants (Table I: 3.99 nM on \(\alpha_9\beta_2\) F117A; 3 nM on \(\alpha_9\beta_2\) V109A; 2.4 nM on \(\alpha_9\beta_2\) V109G) compared with the wild type \(\alpha_9\beta_2\) receptor (55 nM). Moreover, this A10L modification converted PnIA into a potent antagonist at the \(\alpha_9\beta_2\) receptor (Fig. 5B).

Residue 10 in PnIA has previously been shown to be an important determinant of selectivity between \(\alpha_3\beta_2\) and \(\alpha_7\) receptors (8). A comparison of a range of conotoxins with sequence similarity in the second loop showed that the length of the side chain in position 10 correlated with \(\alpha_3\beta_2\) versus \(\alpha_7\) selectivity. This correlation was also found to be valid for GID and PnIA but not for MII, suggesting that MII has an altered binding mode. However, the similar influence of \(\beta_2\) mutants on GID, PnIA, and MII affinity indicate that these \(\alpha_4\beta_2\)-conotoxins orientate similarly within the cleft formed by the \(\alpha\beta\) interface and that the critical position 10 side chain likely faces the \(\beta_2\) subunit in all three \(\alpha\)-conotoxins.

**Shape and Size Complementarity between 4/7 \(\alpha\)-Conotoxins and the Cleft in nAChRs**—A high affinity complex requires either a large contact area between receptor and ligand surfaces with multiple points of interaction or shape complementarity. 

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**FIG. 9. \(\alpha\)-Conotoxin and \(\beta_2\) subunit pharmacophores.** PnIA (A) and MII (B) conotoxins are presented in their docked orientation in the cleft (C). The minimal pharmacophore shown is consistent with mutagenesis results and previous \(\alpha\)-conotoxin alanine scan data. Distances (Å) between residues are measured from the last carbon of the side chain and reported on the vectors in the right-hand panels. Also shown are the equivalent residues for the \(\beta_3\) (green), \(\beta_4\) (blue), and \(\alpha_7\) (red) subunits. Note that Phe-117 is positioned under Glu-59.

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**\(\beta_2\) nAChR Subunit Contributes to \(\alpha\)-Conotoxin Binding**

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tarity that favors the formation of a smaller number of specific interactions (e.g., a ligand bound in a protein cleft). Given their relatively small size, the high affinity complex observed for α-conotoxins suggests that a tight fit between the α-conotoxin and a cleft on the nAChR surface is likely to occur. The α7 nAChR cleft appears to be ~7 Å deep, 13.5 Å long, and 9.5 Å wide (6.6 Å at the narrowest point). These dimensions are almost identical to those found for the α3/α2 nAChR (7.5 Å deep, 14.5 Å long, and 9.8 Å wide (6.8 Å at the narrowest)), and are mostly conserved in shape. For comparison, we measured the dimensions of the N-terminal hydrophobic patch of α-conotoxins. In MII it is 12 Å long (Gly- to Val-7) and 7 Å wide, and in PnIA it is 11.2 Å long (Gly-1 to Pro-7) and 6.5 Å wide, whereas GID has a flexible tail that compiles the measurements (a tail-truncated analogue has similar dimension to PnIA and MII, and the tail could be accommodated by the flexible loop F of nAChR). These comparisons reveal a striking size and shape complementarity between 4/7 α-conotoxins and the nAChR cleft, strongly suggesting a lock-and-key interaction (Fig. 7).

Docking Models of PnIA and MII Interactions with α3/α2 nAChR—To verify that 4/7 α-conotoxins can interact with the nAChR cleft via their conserved hydrophobic patch (residues 6, 7, and 10), docking of PnIA and MII structures onto an α3/α2 nAChR model were simulated using the program GOLD. Analysis of the results from docking simulations confirmed both hypotheses: the small cleft is the binding site for 4/7 α-conotoxins, and the α-conotoxins bind to the α3/α2 nAChR by presenting their conserved N-terminal shape (Fig. 7). Indeed, although an active site radius set to 20 Å would have allowed ligands to dock at other locations around the C-loop, all structures were found docked in the cleft above the βθ10 hairpin. The final orientation of PnIA and MII docked into the receptor cleft presented in Fig. 8 was chosen from the cluster of lowest energy structures produced by GOLD and on the basis of how well it fitted the available experimental data. Both α-conotoxins bind in a similar way to the docking mode found for PnIB at the α7 nAChR (17). It is interesting to note that although MII still binds to α3/α2 from its hydrophobic patch similarly to PnIA, its position in the cleft appears slightly shallower compared with PnIA, as expected given the greater width of its C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7). Interestingly, MII is observed to make additional contacts with the C-terminal half (see Fig. 7).

Point mutations in this cleft revealed a major effect of β2-Leu-119 on α-conotoxin affinity and important influences of β2-Phe-117 and β2-Val-109 on α-conotoxin binding kinetics. Furthermore, we could demonstrate a specific interaction of residue 10 in α-conotoxins with the β2 subunit. Docking simulations of PnIA and MII at α3/α2 confirmed these results by showing a direct interaction between β2 and α-conotoxin residues. These studies indicate that α-conotoxins block the nAChRs by a lock-and-key interaction, where a primary hydrophobic interaction between β2-Leu-119 and the conserved Pro of 4/7 α-conotoxins “locks” the ligand in its cleft, and secondary complementary interactions contribute to ligand selectivity. Based on homology models of nAChRs, the identification of ligand-accessible residues in the cleft (Leu-119, Val-109, and Phe-117) and the determination of a minimal antagonist pharmacophore, a rational approach to the design of subtype-selective nAChR modulators can now be pursued.

Conclusions—We have demonstrated the predictiveness of nAChR homology models based on the AChBP structure and characterized a small cleft as the common binding site for 4/7 α-conotoxins. Point mutations in this cleft revealed a major effect of β2-Leu-119 on α-conotoxin affinity and important influences of β2-Phe-117 and β2-Val-109 on α-conotoxin binding kinetics. Furthermore, we could demonstrate a specific interaction of residue 10 in α-conotoxins with the β2 subunit. Docking simulations of PnIA and MII at α3/α2 confirmed these results by showing a direct interaction between β2 and α-conotoxin residues. These studies indicate that α-conotoxins block the nAChRs by a lock-and-key interaction, where a primary hydrophobic interaction between β2-Leu-119 and the conserved Pro of 4/7 α-conotoxins “locks” the ligand in its cleft, and secondary complementary interactions contribute to ligand selectivity. Based on homology models of nAChRs, the identification of ligand-accessible residues in the cleft (Leu-119, Val-109, and Phe-117) and the determination of a minimal antagonist pharmacophore, a rational approach to the design of subtype-selective nAChR modulators can now be pursued.

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Note Added in Proof—Our studys on 4/7 α-conotoxin binding mode at mammalian nAChRs are strikingly similar to the binding mode determined for 4/7 α-conotoxins co-crystallized with AChBP (Celic, P. H., Kashevverov, I. E., Mordvinsev, D. Y., Hogg, R. C., van Nierop, P., van Elk, R., van Rossum-Fikkert, S. E., Zhmak, M. N., Bertrand, D., Tsetslin, V., Sixma, T. K., and Smit, A. B. (2005) *Nat. Struct. Mol. Biol.*, in press), revealing a remarkable conservation of α-conotoxin binding to nAChRs across different species and subtypes. Interestingly, AChBP has a Met instead of the equivalent Leu-119 in β2 that contributes to the binding of α-conotoxins.

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