Inhibition of Neuronal Nicotinic Acetylcholine Receptor Subtypes by α-Conotoxin GID and Analogues

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α-Conotoxins are small disulfide-rich peptides from the venom of the Conus species that target the nicotinic acetylcholine receptor (nAChR). They are valuable pharmacological tools and also have potential therapeutic applications particularly for the treatment of chronic pain. α-Conotoxin GID is isolated from the venom of Conus geographus and has an unusual N-terminal tail sequence that has been shown to be important for binding to the α4β2 subtype of the nAChR. To date, only four conotoxins that inhibit the α4β2 subtype have been characterized, but they are of considerable interest as it is the most abundant nAChR subtype in the mammalian brain and has been implicated in a range of diseases. In this study, analysis of alanine-scan and truncation mutants of GID reveals that a conserved proline in α-conotoxins is important for activity at the α7, α3β2, and α4β2 subtypes. Although the proline residue was the most critical residue for activity at the α3β2 subtype, Aspβ, Argβ2, and Asnβ4 are also critical at the α7 subtype. Interestingly, very few of the mutations tested retained activity at the α4β2 subtype indicating a tightly defined binding site. This lack of tolerance to sequence variation may explain the lack of selective ligands discovered for the α4β2 subtype to date. Overall, our findings contribute to the understanding of the structure-activity relationships of α-conotoxins and may be beneficial for the ongoing attempts to exploit modulators of the neuronal nAChRs as therapeutic agents.

α-Conotoxins are small, disulfide-rich peptide toxins isolated from the venom of predatory marine snails of the Conus genus and are generally 12–19 amino acids in length (1, 2). These toxins competitively and specifically inhibit muscle and neuronal nicotinic acetylcholine receptor (nAChR)3 subtypes (3) and are valuable tools in understanding the mechanisms involved in ligand-receptor interactions (4). There is much current interest in the study of various neuronal nAChR receptor subtypes implicated in diverse neurological disorders such as Alzheimer disease, epilepsy, and pain (5, 6) and in the regulation of small-cell lung carcinoma (7, 8).

α-Conotoxin GID is a 19-residue peptide isolated from the venom of Conus geographus that has the amino acid sequence IRDγCCSNPACRVNNOHVC (9). Notable features of its sequence include a four-residue extended N-terminal “tail” upstream of the first cysteine, two post-translationally modified residues, γ-carboxyglutamyl acid (Gla) and hydroxyproline (Hyp), and the fact that it lacks an amidated C terminus typical of most α-conotoxins. The three-dimensional structure of GID includes the classic two-loop structural motif of the α-conotoxins, but the N-terminal tail is disordered in solution, as shown in Fig. 1. GID selectively inhibits the α7, α3β2, and α4β2 subtypes of the neuronal nAChR, with high potency, with IC50 values of 4.5, 3.1, and 152 nM, respectively (9). Deletion of the N-terminal four residues Δ(1–4)GID has been shown to significantly decrease activity at the α4β2 receptor, while causing no change in potency at α7 and α3β2 receptors (9). It has been reported that Argβ2 is important for activity at the α4β2 and the α7 receptor but not at the α3β2 receptor.

In this study, we used alanine-scan mutagenesis to identify which GID residues affect potency at α7, α3β2, and α4β2 neuronal nAChRs. The α4β2 receptor subtype is of particular importance because it is widely distributed in the brain and specific ligands have therapeutic potential (10). Additional mutants were synthesized to examine the effect of the unusual N-terminal tail and post-translational modifications present in GID. Mutants were synthesized with truncations in the N-terminal tail, and the γ-carboxyglutamyl acid residue was replaced with a glutamic acid, and the hydroxyproline was replaced with a proline. In addition, the C terminus was amidated as this post-translational modification is present in the majority of α-conotoxins.

To fully understand the effects of mutations, as well as determining their effects on activity, it is crucial to examine influences on structure. Conotoxins are ideally suited to analysis with NMR spectroscopy, and this technique was used for the GID analogues. Specifically, NMR H-α chemical shifts were...
used to identify the presence of any structural differences of the mutants from the native toxin. Because of the significant influence of truncation of the N-terminal tail on \( \alpha 4\beta 2 \) receptor activity (9), the complete three-dimensional structure of the truncated peptide was determined. Furthermore, given the critical role of Arg\(^{12} \) in \( \alpha 7 \) and \( \alpha 4\beta 2 \), the complete solution structure of [R12A]GID was also determined. Overall, the results reveal that although only a few residues are critical for activity at the \( \alpha 7 \) and \( \alpha 3\beta 2 \) nAChR subtypes, few of the mutations studied maintain activity at the \( \alpha 4\beta 2 \) receptor, indicating that the interaction of GID with this nAChR subtype requires much more specific interactions.

**EXPERIMENTAL PROCEDURES**

**Synthesis of GID Mutants**

For peptide synthesis, GID and analogues were manually synthesized by \(-\)butoxycarbonyl chemistry, deprotected, and cleaved from the resin as described previously (11). The HPLC-purified reduced peptides (100 \( \mu M \)) were oxidized in 100 mM ammonium bicarbonate at pH 7.5–8.2 with stirring for 48 h at 4°C. The oxidized peptides were purified by preparative RP-HPLC. GID was quantified in triplicate by amino acid analysis (12) and RP-HPLC using an external reference standard for each peptide. All other mutants were quantified by absorbance using GID as a reference.

**Functional Characterization**

**RNA Preparation**—Plasmids containing cDNA encoding rat \( \alpha 3, \alpha 4, \alpha 7, \beta 2, \) and \( \beta 4 \) nAChR subunits were provided by J. Patrick (Baylor College of Medicine, Houston, TX) and subcloned into the oocyte expression vector pNKS2 (13). Capped cRNAs were synthesized from linearized plasmid cDNAs using the Message Machine kit (Ambion, Austin, TX).

**Expression in Xenopus Oocytes**—Oocytes were prepared as described previously (14, 15). Briefly, oocytes were injected with 50 nl of cRNA (5–50 ng/\( \mu l \)) and kept at 18–19°C in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 5 mM Hepes, pH 7.4) supplemented with 50 mg/liter gentamycin and 5 mM pyruvic acid or 1 mM Ca\(^{2+} \) (Sigma).

**Two-electrode Voltage Clamp Recording**—Two-electrode voltage-clamp recordings were performed on Xenopus oocytes 2–10 days after cRNA injection using holding potentials of --70 or --80 mV. Pipettes were pulled from borosilicate glass (Harvard Apparatus Ltd., Kent, UK) and when filled with 3 mM KCl had resistances below 1.5 megohms. Currents were recorded using either a TEC-05 amplifier (npi electronics, GmbH, Tammi, Germany) or an automated workstation with eight channels in parallel, including drug delivery and on-line analysis (OpusXpressTM 6000A workstation, Molecular Devices, Union City, CA), filtered at 100–200 Hz, and sampled at 300–500 Hz. Recordings were performed in ND96 at room temperature (22–24°C). The half-maximally effective agonist concentration (EC\(_{50} \)) was determined for each of the nAChR subtypes tested. EC\(_{50} \) values of ~300, 30, and 10 \( \mu M \) ACh were obtained for \( \alpha 7, \alpha 3\beta 2, \) and \( \alpha 4\beta 2 \) nAChRs, respectively, which are consistent with those reported previously (16, 17). In this study, 100 \( \mu M \) ACh was used to activate \( \alpha 3\beta 2 \) and \( \alpha 4\beta 2 \) subtypes, and 100 \( \mu M \) ACh or nicotine was used to activate \( \alpha 7 \) nAChRs. This concentration was chosen to be consistent with previous experiments (9). Furthermore, lower ACh concentrations (10–30 \( \mu M \)) used to activate \( \alpha 4\beta 2 \) nAChRs gave similar results for conotoxin inhibition indicating that the agonist concentration did not influence the results. Nicotine was used for the \( \alpha 7 \) nAChRs to avoid the partial agonist effect of choline because of the potential breakdown of ACh. Toxins and agonists were applied as described (15) or by the OpusXpress drug delivery system (18). Peak current amplitudes were measured before and following 180 or 200 s incubation of the GID analogues. To obtain estimates of potency, concentration-response curves were fitted to the pooled data by Equation 1,

\[
\% \text{ response} = 100/(1 + ((\text{toxin})/\text{IC}_{50}^{\alpha}))^{m}
\]  

(Eq. 1)

using Sigmaplot (SPSS Inc. Chicago, IL) or Prism (Graph Pad, San Diego) software, with significant differences between the control GID and the analogues determined by \( t \) test (\( p < 0.05 \)).

**NMR Solution Structures**

**NMR Spectroscopy**—Samples for \(^1\)H NMR measurement contained 0.7–2.5 mM synthetic peptide in 95% H\(_2\)O, 5% D\(_2\)O, or 100% D\(_2\)O at pH 4. Spectra were obtained on a Bruker AMX 500-MHz, Bruker DMX 600-MHz, or Bruker DMX 750-MHz spectrometer at 280 and 287 K. All spectra were acquired in phase-sensitive mode using time proportional phase as described previously (9). Briefly, the homonuclear spectra recorded included DQF-COSY and TOCSY, using a MLEV17 spin lock sequence with an isotropic mixing time of 80 ms, and ECOXY and NOESY with mixing times of 150, 250, and 350 ms. In DQF-COSY and ECOXY experiments, the water resonance was suppressed by low power irradiation during the relaxation delay. For the TOCSY and NOESY experiments, water suppression was achieved using a modified WATERGATE sequence. Two-dimensional spectra were generally collected over 4096
Structure-Activity of α-Conotoxin GID

data points in the f2 dimension and 512 increments in the f1
dimension over a spectral width corresponding to 12 ppm. For
identification of slowly exchanging amides, a series of one-di-

mensional and TOCSY spectra were run immediately after dis-
solving the sample in D2O. All spectra were processed using
XWINNMR (Bruker). The f1 dimension was zero-filled to 2048
real data points with the f1 and f2 dimensions being multiplied
by a sine-squared function shifted by 90° prior to Fourier trans-
formation. Processed spectra were analyzed and assigned using
the program XEASY (19). Spectra were assigned using the se-
quential assignment protocol (20).

Structure Calculations—Cross-peaks in NOESY spectra
recorded in 95% H2O, 5% D2O with mixing times of 250 ms
were integrated and calibrated in XEASY, and distance con-
straints were derived using DYANA (21). Backbone dihedral
angle restraints were derived from 3\(^J\)HNNH coupling constants
measured from line shape analysis of antiphase cross-peak
splitting in the DQF-COSY spectrum. Angles were restrained
to -120 ± 30° for 3\(^J\)HNNH > 8.5 Hz and to -60 ± 30° for
3\(^J\)HNNH < 5 Hz. Stereospecific assignments of β-methylene pro-
tons and χ1 dihedral angles were derived from 3\(^J\)\(\alpha\)β coupling
constants, measured from ECOXY spectra, in combination with
NOE peak intensities. Slowly exchanging amide protons iden-
tified by D2O exchange experiments were used in conjunction
with preliminary structures to determine hydrogen bonds. In
cases where hydrogen bonds could be unambiguously assigned,
appropriate distance restraints were included in the structure
calculations. Preliminary structures were calculated using a
torsion angle simulated annealing protocol within DYANA and
final structures calculated using simulated annealing and
energy minimization protocols within CNS version 1.0 as
described previously (9). The starting structures were gener-
ated using random φ, ψ dihedral angles and energy-minimized
to produce structures with the correct local geometry. A set of
50 structures was generated by a torsion angle simulated
annealing protocol (22). This protocol involves a high temper-

ature phase comprising 4000 steps of 0.015 ps of torsion angle
dynamics during which the temperature is lowered to 0 K,
and finally an energy minimization phase comprising 5000
steps of Powell minimization. Structures consistent with
restraints were subjected to further molecular dynamics and
energy minimization in a water shell, as described by Linge and
Nilges (23). The refinement in explicit water involves the fol-

lowing steps. First heating to 500 K via steps of 100 K, each
comprising 50 steps of 0.005 ps of Cartesian dynamics. Second,
2500 steps of 0.005 ps of Cartesian dynamics at 500 K before a
cooling phase where the temperature is lowered in steps of 100
K, each comprising 2500 steps of 0.005 ps of Cartesian dynam-
ics. Finally, the structures were minimized with 2000 steps of
Powell minimization. Structures were analyzed using Promotif
and Procheck (24, 25).

RESULTS
Synthesis and Oxidative Folding of Mutants—The GID
mutants were synthesized using solid phase peptide synthesis
with t-butoxycarbonyl chemistry. A list of the sequences of the
analogues is given in Table 1. All mutants folded predominantly
into a single isomer in ammonium bicarbonate buffer as deter-
mined by RP-HPLC. To compare the effects of the synthetically
mutated peptides on activity, we determined their IC50 values
for the three subtypes for activity at

| TABLE 1  |
|---------|
| Sequences of GID and analogues |
| Peptide         | Sequence |
|-----------------|----------|
| GID             | TRDCCSNPACRHNNOHVC |
| [γE]GID (GID*)  | TRDCCSNPACRHNNOHVC |
| [I1A]GID*       | ARDCCSNPACRHNNOHVC |
| [R2A]GID*       | TADCCSNPACRHNNOHVC |
| [D3A]GID*       | TFAECCSNPACRHNNOHVC |
| [γA]GID         | TRDCCSNPACRHNNOHVC |
| S7A[GID*       | IRDCCSNPACRHNNOHVC |
| N8A[GID*       | IRDCCSNPACRHNNOHVC |
| P9A[GID*       | IRDCCSNPACRHNNOHVC |
| R12A[GID*       | IRDCCSNPACRHNNOHVC |
| V13A[GID*       | IRDCCSNPACRHNNOHVC |
| N14A[GID*       | IRDCCSNPACRHNNOHVC |
| N15A[GID*       | IRDCCSNPACRHNNOHVC |
| O16A[GID*       | IRDCCSNPACRHNNOHVC |
| O16P[GID*       | IRDCCSNPACRHNNOHVC |
| H17A[GID*       | IRDCCSNPACRHNNOHVC |
| V18A[GID*       | IRDCCSNPACRHNNOHVC |
| R2A[11]GID      | ADECCSNPACRHNNOHVC |
| D3A[11-2]GID*   | ACSCSNPACRHNNOHVC |
| [1-3]GID*       | ECCRCCSNPACRHNNOHVC |
| [γA,1-3]GID     | AECSCSNPACRHNNOHVC |
| [1-4]GID        | CCSSNPACRHNNOHVC |

* [R12A]GID was synthesized with a γ-carboxyglutamate at position 4, in contrast
to the other mutants, because it was obtained from the study by Nigge et al. (9).

Functional Assays—α-Conotoxin GID antagonized the α7, α3β2, and α4β2 nAChRs with IC50 values of 5.1, 3.4, and 128.6
nm, respectively, which is consistent with the data reported pre-
viously (9). The activity at the three different nAChRs was not
significantly changed with the substitution of a glutamic acid
residue in the position of the γ-carboxyglutamyl acid. This ana-
logue ([γE]GID), resulted in IC50 values of 5.9, 2.9, and 56.8 nm
for the α7, α3β2, and α4β2 subtypes, respectively, as shown in
Fig. 2. Thus, all other (alanine-substituted) analogues were syn-
thesized with glutamic acid at position 4 with the exception of
R12A, which was obtained from a previous study where γ-car-
boxyglutamyl was used (9). [γE]GID is referred to hereafter as
GID* to simplify the naming scheme of the analogues.

Analysis of Alanine Replacement/Truncations—Truncation
of the N-terminal tail had been shown previously to cause a
decrease in activity at the α4β2 subtype but not at the α7 and
α3β2 subtypes (9), and thus it was of interest to examine the
effects of alanine mutations of the tail as well as N-terminally
truncated variants. For the series of alanine mutants, the most
significant differences in activity were observed at the α4β2
subtype, as shown in Table 2. Replacement of Ile1 and Glu4 with
Ala had relatively small but significant effects on the IC50 values
at the α4β2 subtype. By contrast, replacement of residues 2 and
3 with Ala led to no detectable activity at concentrations up to 1
μM. At the α7 subtype [D3A]GID* had decreased activity. Trun-
cating the N terminus sequentially by 1–4 residues also resulted in
inactive analogues at the α4β2 subtype, whereas in general only
minor variations in the IC50 values were observed at the α7 and
α3β2 subtypes (Table 2). Interestingly, [γA]GID exhibited
increased potency at αβ2 subtype, whereas the decreased
potency of [γA,1-3]GID highlighted the importance of the first
three residues for activity at α7, α3β2, and α4β2.
The α4β2 receptor proved to be the greatest discriminator of activity, with the majority of mutations showing a 10-fold or complete loss of activity when compared with GID. The only mutant without a significant influence on activity was [V13A]GID*. The mutations that led to a loss in activity included the amidation of the C terminus, a common post-translational modification observed in α-conotoxins. The significant reduction in potency demonstrated by several residues throughout the N-terminal tail and the two inter-cysteine loops suggest that the interaction of the toxin with the α4β2 receptor is highly specific.

NMR Secondary Shift Analysis of GID Mutants—All of the alanine mutants were analyzed by NMR, and the H-α chemical shifts were determined and compared with GID. The differences between the H-α chemical shifts and random coil values are shown in Fig. 3. In general, the mutants overlay closely with GID, and therefore no major changes in secondary structure were observed with the alanine substitutions. This result suggests that all the mutants have a well defined helix, as is observed for almost all α-conotoxins (26, 27). The greatest chemical shift variation is observed for the N-terminal tail region for the truncation mutants.

Structural Characterization of [Δ1–4]GID and [R12A]GID—Analysis of the 250-ms NOESY spectra (750 MHz, 287 K) of the two mutants [Δ1–4]GID and [R12A]GID using the program XEASY allowed the assignment of each spin system to a specific amino acid. All non-intraresidual peaks were subsequently assigned using the sequential assignment method (20). Interproton distance restraints were derived from the NOE intensities and used in structure calculations using a torsion angle simulated annealing protocol within DYANA. Preliminary structures were analyzed to resolve spectral ambiguities and to facilitate the introduction of new restraints. For [Δ1–4]GID, a set of restraints consisting of 149 NOE derived distances and 5 dihedral angle restraints was used in the final calculations. These restraints included 57 sequential, 30 medium range, 12 long range, and 50 intra-residue distances, and 5 φ angle restraints (Cys2, Ser3, Ala6, Asn10, and Asn11). χ1 restraints were derived on the basis of coupling constants and NOE intensities from a 150-ms NOESY spectrum. There were no restraints included for hydrogen bonds as none were identified in preliminary structures or in slow exchange experiments.

For [R12A]GID, a set of restraints consisting of 173 NOE derived distances and 17 dihedral angle restraints was used in the final calculations. These restraints included 88 sequential, 65 medium range, 8 long range, and 12 intra-residue distances, 10 φ angle restraints (Asp3, Gla4, Cys5, Cys6, Ser7, Asn8, Ala10, Ala12, Val13, and His17), and 7 χ1 angle restraints (Cys5, Cys6, Asn8, Cys7, Val13, Asn14, and Cys19). There were also two restraints included for one hydrogen bond identified in preliminary structures.

Restrains defining the globular disulfide bond connectivity (Cys1-Cys10 and Cys4-Cys14) were included in the structure calculations for both [Δ1–4]GID and [R12A]GID based on the chemical shift analysis, which indicates that these peptides have a native (i.e. “globular”) fold. Previous studies have shown that the alternative “ribbon” or “beads” disulfide isomers of α-conotoxins display clearly different chemical shifts (28, 29).
**TABLE 2**

**Electrophysiology results for GID and its analogues**

$IC_{50}$ values (mean ± S.E.) (nM) and Hill slopes ($n_H$) for inhibition of agonist-evoked currents through α7, α3β2, and α4β2 nAChR subtype combinations were expressed in *Xenopus* oocytes.

| Mutants          | α7  |        | α3β2 |        | α4β2 |        |
|------------------|-----|--------|------|--------|------|--------|
|                  | $IC_{50}$ | Hill slope | n | $IC_{50}$ | Hill slope | n | $IC_{50}$ | Hill slope | n |
| GID              | 5.1 ± 1.3 | 1.3 | 3 | 3.4 ± 0.5 | 1.3 | 3 | 128 ± 13.1 | 1.2 | 4 |
| y1E[GID]         | 5.9 ± 0.6 | 1.3 | 3 | 2.9 ± 0.6 | 1.1 | 3 | 56.8 ± 13.2 | 1.0 | 3 |
| y1E,11A[GID]     | 5.9 ± 1.6 | 0.8 | 4 | 15.5 ± 2.2 | 1.2 | 6 | 255.9 ± 57.2 | 1.3 | 9 |
| y1E,12A[GID]     | 12.5 ± 4.4 | 1.0 | 4 | 97.6 ± 28.4 | 0.9 | 5 | NA         | 4 | 4 |
| y1E,3A[GID]      | 42.1 ± 10.9 | 1.7 | 3 | 69.8 ± 16.5 | 1.2 | 5 | NA         | 4 | 4 |
| y1A[GID]         | 6.1 ± 2.8 | 1.2 | 3 | 3.0 ± 0.6 | 1.7 | 3 | 52.0 ± 16.5 | 1.0 | 3 |
| y1E,57A[GID]     | 12.8 ± 1.5 | 1.4 | 3 | 2.5 ± 0.3 | 2.2 | 4 | 429.9 ± 82.6 | 1.2 | 3 |
| y1E,88A[GID] >30 * | | | | | | | |
| y1E,19A[GID]     | 90.5 ± 22.6 | 1.5 | 3 | 172.6 ± 50.1 | 1.8 | 4 | NA         | 3 | 3 |
| y1E,12A[GID]     | 48.8 ± 4.2 | 1.2 | 3 | 10.4 ± 0.7 | 1.2 | 3 | NA         | 3 | 3 |
| y1E,13A[GID]     | 11.2 ± 4.0 | 0.7 | 3 | 0.6 ± 0.1 | 1.0 | 3 | 112.5 ± 32.1 | 1.0 | 3 |
| y1E,14A[GID]     | 51.4 ± 6.8 | 1.1 | 3 | 1.3 ± 0.5 | 1.0 | 3 | NA         | 3 | 3 |
| y1E,15A[GID]     | 6.0 ± 1.0 | 1.3 | 3 | 3.3 ± 0.2 | 1.1 | 3 | NA         | 3 | 3 |
| y1E,16A[GID]     | 13.0 ± 4.4 | 0.9 | 3 | 13.5 ± 2.1 | 1.2 | 3 | NA         | 3 | 3 |
| y1E,16P[GID]     | 8.4 ± 1.9 | 0.8 | 3 | 6.3 ± 0.8 | 1.7 | 3 | 571.7 ± 59.6 | 1.1 | 3 |
| y1E,17A[GID]     | 5.0 ± 1.9 | 0.9 | 3 | 2.7 ± 0.2 | 2.2 | 3 | 602.0 ± 75.1 | 0.9 | 3 |
| y1E,18A[GID]     | 7.9 ± 2.9 | 0.8 | 3 | 40.8 ± 8.0 | 1.2 | 8 | NA         | 3 | 3 |
| y1E,2A,11[A]GID  | 14.4 ± 4.2 | 1.5 | 3 | 19.6 ± 4.2 | 1.0 | 6 | NA         | 4 | 4 |
| y1E,3A,31–2[GID] | 17.5 ± 7.2 | 1.4 | 3 | 32.5 ± 9.1 | 1.2 | 5 | NA         | 4 | 4 |
| y1E,1–3[GID]     | 13.0 ± 1.9 | 1.8 | 4 | 61.2 ± 15.4 | 1.4 | 5 | NA         | 4 | 4 |
| y1E,1–3–5[GID]   | 100.3 ± 8.3 | 0.9 | 4 | 177.7 ± 36.1 | 1.1 | 5 | NA         | 4 | 4 |
| y1E,GID-NH2      | 2.0 ± 0.1 | 1.8 | 4 | 1.7 ± 0.1 | 1.7 | 4 | NA         | 3 | 3 |

* Where $IC_{50}$ values were unable to be determined, estimations are represented as > the highest concentration tested. NA denotes a nonactive analogue at concentrations ≤1 μM. $IC_{50}$ values denoted in boldface are significantly different from the corresponding GID control (t test; p < 0.05).

**FIGURE 3. Comparison of the chemical shifts of GID with the mutants.** The H-α chemical shifts of GID (9) are compared with the mutants. Four separate graphs are shown for clarity.

In the final round of structure calculations, the distance, angle, and disulfide restraints were used to calculate a family of 50 structures, using a torsion angle simulated annealing protocol. Structures consistent with the restraints were subjected to further molecular dynamics and energy minimization in a water shell.

**Description of the Three-dimensional Structures of [Δ1–4]GID and [R12A]GID**—A family of the 20 lowest NOE energy structures was chosen from the final set of 50 structures to represent the solution structures of [Δ1–4]GID and [R12A]GID, respectively. These structures had no distance violations greater than 0.3 Å and no dihedral angle violations.
includes residues 8–13. Hydrogen bonds between Pro9(OH)–Val13(HN) were deduced from analysis of preliminary structures and slow exchange data and were explicitly included in the structure calculations. At the N terminus, the poor definition of residues 1–3, also seen in the wild type peptide GID, may be associated with flexibility of this region. Residues 4–7 are involved in a type I β-turn, whereas residues 14–17 are involved in a type II β-turn. The structure statistics are given in supplemental Table 1.

A surface of [R12A]GID also illustrates a distinct hydrophobic face comprising residues Pro9, Ala10, Ala12, Val13, Hyp16, and Val17. The hydrophilic residues on the surface include Ser7, Asn8, Asn14, and Asn15. There are also three charged residues on the surface of the molecule. Three (Arg2, Asp3, and Gla4) are part of the disordered N-terminal tail. The loss of the positive charge because of the R12A mutation adjacent to the hydrophobic face of the molecule may be involved in the loss of potency at the α4β2 receptor.

**DISCUSSION**

In this study, we synthesized and analyzed a series of alanine and truncation mutants of α-conotoxin GID to gain an understanding of the importance of the N-terminal tail and the individual residues on both structure and activity at the α7, α3β2, and α4β2 subtypes of the neuronal nAChR. To our knowledge, this study has provided the first significant information on α-conotoxin interaction with the α4β2 subtype of the nAChR in addition to significant information on the factors influencing activity at the α7 and α3β2 nAChR subtypes. The conserved proline residue in the α-conotoxins was found to be critical for activity at all subtypes tested, and although many mutations produced significant effects at α3β2 nAChRs, very few mutations were tolerated at the α4β2 subtype.

Oxidation reactions of the mutants produced predominantly the globular (native) isomers unlike oxidation reactions of ImI, PnIA, and Nib, where large amounts of all three possible disulfide bond isomers were obtained (31). Thus, it appears that formation of the globular connectivity of the GID mutants is highly favored under the conditions employed in this study. Furthermore, analysis of the GID mutants with NMR spectroscopy indicates that the GID scaffold is amenable to sequence variations as no significant changes in chemical shift are observed for the majority of the peptide backbone. This was confirmed by determination of the three-dimensional structure of [R12A]GID, which was found to be similar to native GID. Truncations in the N-terminal tail produced significant chemical shift variations in the tail, but again the overall fold of the molecule in the inter-cysteine loops is similar to the native peptide. Interestingly, despite the overall fold of truncated α-conotoxin [Δ1–4]GID being similar to the native peptide, it is a less well defined molecule than GID, especially in loop 2 of the structure with an r.m.s.d. of 1.16 Å compared with 0.34 Å of GID over equivalent residues. Truncation of GID also caused a lack of slowly exchanging amide protons. These results indicate that the N-terminal tail of GID has a structural role in maintaining the α-conotoxin fold in GID that is not apparent in the majority of α-conotoxins, which maintain a single preferred

**FIGURE 4.** The three-dimensional structures of [Δ1–4]GID and [R12A]GID. Overlays of the 20 lowest energy structures are shown on the left, and a ribbon diagram highlighting the secondary structure is shown on the right. The disulfide bonds are shown in ball and stick format and are labeled with roman numerals. The helices are represented as thickened ribbons. A, [Δ1–4]GID. B, [R12A]GID. Both structures are showing the typical structural features of α-conotoxins.

**Structure-Activity of α-Conotoxin GID**

The surface of [Δ1–4]GID illustrates a distinct hydrophobic face comprising residues Pro5, Ala6, and Val9 and a second hydrophobic patch Hyp12, His13, and Val14. The hydrophilic residues on the surface include Ser7, Asn8, Asn14, and Asn15. There is also one positively charged residue on the surface of the molecule (Arg8) situated adjacent to the two hydrophobic faces.

The structures of [R12A]GID were well defined with the exception of the N-terminal tail, which had few experimental restraints defining this region. The mean r.m.s.d. over residues 4–19 was 0.35 ± 0.12 Å for the backbone atoms and 1.10 ± 0.35 Å for the heavy atoms. The family of structures obtained, superimposed over the backbone atoms of residues 4–19, is shown in Fig. 4. Analysis with PROMOTIF indicates that the α-helix...
Structure-Activity of α-Conotoxin GID

structure in solution despite the absence of an extended N-terminal tail (26).

Differences in biological activity were observed for several of the GID mutants relative to the native peptide. The NMR analysis indicates these differences are not a result of overall changes to the secondary structure present but rather the individual residues appear to have a functional role. We have previously shown that the N-terminal tail is important for activity at the α4β2 subtype, and in this study we have been able to further define the tail residues that are important for activity. In particular, the charge state of the N-terminal tail appears to play a critical role in activity at the α3β2 and α4β2 nAChRs as replacement of either Arg2 or Asp3 significantly reduces or abolishes activity, whereas replacement of Ile3 with alanine shows a significant decrease at both. No significant losses in activity are observed for the alanine mutants in the tail region for the α7 nAChR subtypes with the exception of [D3A]GID-

In contrast to the removal of the charges at residues 2 and 3, removal of the negative charges exhibited by substitution of Gla4 with either a Glu or Ala residue did not decrease the efficacy at any of the subtypes tested. In fact, both [γ4A]GID and [γ4E]GID (GIDγ) exhibited a statistically significant increase in potency at the α4β2 receptors when compared with GID. The post-translationally modified Gla residue therefore does not appear to be functionally important and may be present to increase stability of the peptide to proteolytic attack.

Although the influence of the tail at α7 and α3β2 subtypes is not as significant as the α4β2, it is interesting to note that [γ4E,Δ1–3]GID and [γ4A,Δ1–3]GID display significant losses of activity across all subtypes. These results indicate the N-terminal residues also play a role in binding to the α7 and α3β2 subtypes. The complexity of the structure-activity relationships of GID is evident from these results and may be due to the disorder inherent in the native molecule. For instance, it is not clear why the deletion in [Δ1–4]GID has no effect on the α7 receptor (9), whereas [D3A]GID
gamma
delta

Analysis of the alanine mutants of the two intercysteine loops of GID revealed that only D3A and P9A exhibited a >10-fold loss of activity at all three nAChR subtypes relative to native GID. The residues important for binding at the three subtypes tested are mapped onto the native GID structure in Fig. 5. Three of the four residues important for binding to the α7 subtype are on a single face of the molecule, indicating this face is important for activity. The striking finding from this study was that there are a large number of residues that resulted in significant loss of activity at the α4β2 subtype upon mutation to an alanine. Only mutation of the Gla4 with either an alanine or a glutamic acid and mutation of Val13 to alanine maintained activity. Interestingly, replacement of the Gla4 residue with either a glutamic acid or alanine significantly increased the activity at the α4β2 subtype indicating that negative charges at this position interfere with binding. Our original study revealed that the N-terminal tail was important for activity at the α4β2 subtype, but it is now clear that the majority of residues are involved in binding and that specificity is not limited to the N-terminal tail. In support of this, the amidation of the C terminus had no or positive effects on the potency of α7 and α3β2 subtypes but strongly reduced the potency at the α4β2 subtype. Because an amidated C terminus is common to many native α-conotoxins, this might explain the difficulty in discovering α4β2-selective α-conotoxins.

The importance of the Pro residue for binding is consistent with previous studies on related α-conotoxins. A proline residue in the first intercysteine loop of the α-conotoxins is highly conserved across the family and appears to be extremely important for binding to the nAChR (31, 32). Pro9 of GID is equivalent to the highly conserved proline in the α-conotoxin family, which we have shown is important for activity at all three subtypes tested. In a previous study on α-conotoxin MII, it was also shown to be important (32). An alanine scan carried out on α-conotoxin MII revealed that Asn5, Pro6, and His12 were the major determinants in this conopeptide for potency at the α3β2 nAChR (32). This study also examined the structural integrity of the mutants with circular dichroism and protein modeling, and it was reported that all three mutants were structurally similar to the native MII, and therefore the loss in toxin potency is not because of a change in secondary structure. This lack of structural change is consistent with this study on GID. The importance of the proline has also been highlighted in another study demonstrating that despite the IC50 values for both α-conotoxins 1m1 and 1m11 being similar, the presence of the proline at position 6 is essential for competitive binding with α-bungarotoxin at the α7 receptor (33). This finding suggests
that the binding site of lm1L (without the Pro) is different to that of lm1 and α-bungarotoxin.

With the structure determination of the acetylcholine-binding protein, a homologue of the nAChR, new insights into the binding of conotoxins has been gained (34–36). It appears that the α-conotoxin is buried deep within the ligand-binding site and does not change conformation, relative to the solution structure, upon binding (34). Furthermore, the binding of α-conotoxins is dominated by hydrophobic interactions. Given the striking results for the binding of GID to the α4β2 subtype, it is of considerable interest to examine the structural aspects of the binding of peptides to the α4β2 subtype. Direct evidence regarding this interaction is difficult given the large membrane-bound nature of the receptor, but clues may be gained from molecular modeling techniques (37).

In summary, analysis of alanine-scan and truncation mutants of GID indicate that Pro9 has a significant functional role in binding to all neuronal nAChR subtypes. Interestingly, multiple contacts occur upon binding of GID to the α4β2 subtype as mutations of several residues significantly decrease activity. In general, a limited number of residues have been found to be important for binding of α-conotoxins to neuronal nAChRs, but the current results suggest that the α4β2 nAChR subtype requires much greater specificity to interact. Furthermore, the N-terminal residues appear to have a structural role in stabilizing the fold of GID. This study highlights the complexity of the structure-activity relationships of this class of molecules and provides valuable information on the binding of ligands to the neuronal nAChR and in particular to the α4β2 subtype. This information may be beneficial for the ongoing attempts to exploit modulators of neuronal nAChRs as therapeutic agents.

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