2003

Isolation, structure, and activity of GID, a novel α4/7-conotoxin with an extended N-terminal sequence

Annette Nicke
University of Queensland

Marion L. Loughnan
University of Queensland

Emma L. Millard
University of Queensland

Paul F. Alewood
University of Queensland

David J. Adams
University of Queensland, djadams@uow.edu.au

See next page for additional authors

Publication Details

Research Online is the open access institutional repository for the University of Wollongong. For further information contact the UOW Library: research-pubs@uow.edu.au
Isolation, structure, and activity of GID, a novel α4/7-conotoxin with an extended N-terminal sequence

Abstract
Using assay-directed fractionation of Conus geographus crude venom, we isolated α-conotoxin GID, which acts selectively at neuronal nicotinic acetylcholine receptors (nAChRs). Unlike other neuronally selective α-conotoxins, α-GID has a four amino acid N-terminal tail, γ-carboxyglutamate (Gla), and hydroxyproline (O) residues, and lacks an amidated C terminus. GID inhibits α7 and α3β2 nAChRs with IC50 values of 5 and 3 nM, respectively and is at least 1000-fold less potent at the α1β1γδ, α3β4, and α4β4 combinations. GID also potently inhibits the α4β2 subtype (IC50 of 150 nM). Deletion of the N-terminal sequence (GIDΔ1-4) significantly decreased activity at the α4β2 nAChR but hardly affected potency at α3β2 and α7 nAChRs, despite enhancing the off-rates at these receptors. In contrast, Arg12 contributed to α4β2 and α7 activity but not to α3β2 activity. The three-dimensional structure of GID is well defined over residues 4-19 with a similar motif to other α-conotoxins. However, despite its influence on activity, the tail appears to be disordered in solution. Comparison of GID with other α4/7-conotoxins which possess an NN(P/O) motif in loop II, revealed a correlation between increasing length of the aliphatic side-chain in position 10 (equivalent to 13 in GID) and greater α7 versus α3β2 selectivity.

Disciplines
Medicine and Health Sciences

Publication Details

Authors
Annette Nicke, Marion L. Loughnan, Emma L. Millard, Paul F. Alewood, David J. Adams, Norelle L. Daly, David J. Craik, and Richard J. Lewis

This journal article is available at Research Online: http://ro.uow.edu.au/ihmri/693
Isolation, Structure, and Activity of GID, a Novel α4/7-Conotoxin with an Extended N-terminal Sequence*

Received for publication, October 8, 2002
Published, JBC Papers in Press, November 4, 2002, DOI 10.1074/jbc.M210280200

Annette Nickels, Marion L. Loughnan, Emma L. Millard, Paul F. Alewood, David J. Adams, Norelle L. Daly, David J. Craik, and Richard J. Lewis

From the Institute for Molecular Bioscience and School of Biomedical Sciences, University of Queensland, Brisbane, Queensland 4072, Australia

Using assay-directed fractionation of Conus geographus crude venom, we isolated α-conotoxin GID, which acts selectively at neuronal nicotinic acetylcholine receptors (nAChRs). Unlike other neurally selective α-conotoxins, α-GID has a four amino acid N-terminal tail, γ-carboxyglutamate (Gla), and hydroxyproline (O) residues, and lacks an amidated C terminus. GID inhibits α7 and α3β2 nAChRs with IC50 values of 5 and 3 nM, respectively and is at least 1000-fold less potent at the α1β2γδ, α3β4, and α4β4 combinations. GID also potently inhibits the α4β2 subtype (IC50 of 150 nM). Deletion of the N-terminal sequence (GID1–4) significantly decreased activity at the α3β2 nAChR but hardly affected potency at α3β2 and α7 nAChRs, despite enhancing the off-rates at these receptors. In contrast, Arg3 contributed to α4β2 and α7 activity but not to α3β2 activity. The three-dimensional structure of GID is well defined over residues 4–19 with a similar motif to other α-conotoxins. However, despite its influence on activity, the tail appears to be disordered in solution. Comparison of GID with other α4/7-conotoxins which possess an NN(P/O) motif in loop II, revealed a correlation between increasing length of the aliphatic side-chain in position 10 (equivalent to 13 in GID) and greater α7 versus α3β2 selectivity.

Neuronal nicotinic acetylcholine receptors (nAChR) represent important targets for the development of novel drugs for the treatment of pain and various disorders of the central nervous system (1). To date, eight α and three β subunits (α2–α7, α9, α10, β2–β4) of the nAChRs have been cloned from sensory and neuronal mammalian cells (2–4). For the α7 and α9 subunits, it has been shown that they need no additional subunits to form functional ion channels upon heterologous expression. All other α subunits, however, require at least the co-expression of one β subunit, or another α subunit in the case of α10. Ternary combinations of two different α and one β subunit or two different β and one α subunit, and even quaternary combinations have been described (5, 6). This diversity of subunit combinations has the potential to generate a wide range of receptor subtypes with different pharmacological and functional properties. To help unravel which native neuronal nAChR subunit combinations are responsible for specific physiological functions, additional selective inhibitors are required.

Conotoxins are small disulfide-rich peptides from the venom of the predatory marine snails of the genus Conus. These mini-proteins have proved to be valuable tools for investigating the structure and function of ligand- and voltage-gated ion channels. α-Conotoxins are competitive antagonists of acetylcholine (ACh) binding to the nAChR (7). The α-conotoxins described so far are among the most selective inhibitors to be identified (Fig. 1). They are typically 12–18 amino acids long, contain a conserved Pro in loop I, and are folded by two disulfide bonds connecting Cys1–Cys5 and Cys2–Cys4. Based on the number of amino acids between the second and third cysteine residues (loop I) and the third and fourth cysteine residues (loop II) they are divided into α3/5, α4/7, α4/6, and α4/3 structural subfamilies (8). The α3/5-conotoxins are selective for the muscle-type nAChR, while most α4/7- and α4/6-conotoxins are selective for neuronal nAChRs. An exception is the α4/7-conotoxin El, which binds to the β δ interface of the muscle-type nAChR (9). The α4/3-type characterized by ImI is α7-selective (10). The three-dimensional structures of different neuronal-specific and muscle-specific α-conotoxins have been determined by NMR (11–15) and x-ray structural analysis (16–19). The α4/7-conotoxins and ImI share similar backbone conformations and a rigid hydrophobic core (14), suggesting that their different specificities for nAChR subtypes arise from the different amino acid side-chains projecting from this conserved scaffold.

The α4β2 nAChRs together with the α7 subtype are the most abundant nAChRs in mammalian brain. Knockout studies in mice have revealed an important role for α4 and β2 in pain and cognition (20) but further investigations of its role are limited by the lack of α4β2-selective inhibitors. A primary goal of this study was to identify new α-conotoxins active at the α4β2 subtype. In this report, we describe the isolation and characterization of α4/7-conotoxin GID. GID possesses a novel four amino acid N-terminal tail and an Arg in position 12 that contribute to α4β2 selectivity. The NMR structure of GID was determined to define the location of structural features that contribute to the selectivity of GID.
**Isolation and Structure-Activity Studies of α-GID**

**EXPERIMENTAL PROCEDURES**

**Isolation, Purification, and Sequencing of GID**

**Extraction of Crude Venom—**Nine specimens of Conus geographus were collected from the Great Barrier Reef, Australia. Crude venom duct contents were extracted with 30% acetonitrile/water acidified with 0.1% trifluoroacetic acid and centrifuged. Soluble material (120 mg) was lyophilized and stored at −20 °C prior to use.

**Isolation and Purification of GID—**A portion of the crude venom extract (40 mg) was fractionated on a semipreparative RP-HPLC column (10-μm C18, Vydac) eluted at 3 ml/min with a linear gradient of 0–80% solvent B over 80 min (solvent A, 0.1% trifluoroacetic acid; solvent B, 90% acetonitrile, 0.09% trifluoroacetic acid). Inhibition in a functional nAChR assay (see below) identified an active 1-min fraction eluting at −21 min (Fig. 2A), which was further purified by RP-HPLC employing a linear gradient of 0–25% B over 25 or 45 min (5-μm C18, 46 × 250 mm Zorbax or Jupiter columns at 1 ml/min or a SH300 3.5-μm C18, 2.1 × 50 mm Zorbax column at 0.2 ml/min eluted with solvent A, 0.05% trifluoroacetic acid; solvent B, 90% acetonitrile and 0.045% trifluoroacetic acid). An active peptide detected at 214 nm was collected and sequenced after reduction and alkylation with maleimide (see below). Venom was further characterized by LC/MS analysis using a UPLC-Q-TOF mass spectrometer (ACQ-4200–2200) to monitor the eluant from a 5-μm C18, 2.1 × 150 mm Zorbax column eluted with 0–60% B over 60 min (A, 0.1% formic acid; B, 90% aqueous acetonitrile, 0.09% formic acid).

**Sequencing—**The purified peptide (~20 pmol) was reduced in the presence of 10 mM TCEP and 50 mM ammonium acetate, pH 4.5 (37 °C for 1 h) before alkylation in the added presence of 20 mM maleimide (37 °C for 1 h). The alkylated peptide was repurified by RP-HPLC prior to sequence analysis by Edman chemistry on a 492–01 HT model Procise protein sequencer (see Fig. 1). Stock solutions of 100 mM Tris(2-carboxyethyl)-phosphine hydrochloride (Pierce), and maleimide (Aldrich) were prepared in 0.1 M ammonium acetate pH 4.5 and in 10% acetonitrile, respectively, and stored at −20 °C.

**Peptide Synthesis**

GID and analogues were manually assembled by Boc chemistry, deprotected, and cleaved from resin as described previously (21). Amino acid side-chain protection was as follows: Arg (Tos), Asn(Xan), Asp(O-cHex), Cys(sMeBzl), GlcA(OcxH), His (DNP), Hyp(Bzl), and Ser(Bzl).

**RESULTS**

**Functional Characterization of GID**

**RNA Preparation—**Plasmids containing cDNA encoding rat α3, α4, α7, β2, and β4 nACHR subunits were provided by J. Patrick (Baylor College of Medicine, Houston, TX) and subcloned into the oocyte expression vector pNKS2 (23). cDNAs for the mouse α1, β1, γ, and δ subunits of the muscle nAChR were provided by Dr. V. Witzemann (Max-Planck Institute for Medical Research, Heidelberg, Germany). Capped cRNAs were synthesized from linearized plasmid cDNAs using the Message Machine Kit (Ambion, Austin, TX).

**Expression in Xenopus Oocytes—**Oocytes were prepared as previously described (24), injected with 50 nl of cRNA (5–50 ng/μl), and kept at 19 °C in ND96 (96 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 5 mM Hepes at pH 7.4) supplemented with 50 μg/ml of gentamicin (Sigma Chemical Co.).

**Two Electrode Voltage-Clamp Recording—**Two electrode voltage-clamp recordings were performed in oocytes 2–10 days after cRNA injection at a holding potential of −70 mM. Pipettes were pulled from borosilicate glass (Harvard Apparatus Ltd., Kent, England) and filled with 3 mM KCl. Resistances were below 1 MΩ. Membrane currents were recorded using a two electrode virtual ground circuit on a GeneClamp 500B amplifier (Axon Instruments Inc., Union City, CA), filtered at 200 Hz and digitized at 1 kHz using a Digidata 1322A interface and v8.2 Clampex software (Axon Instruments Inc.). Recordings were performed in ND96 at room temperature. 1 μM ACh (Sigma) was used to activate α4β2, α1β1γδ nAChRs, 100 μM ACh was used to activate α3β4, and α4β2 nAChRs, and 100 μM nicotine (Sigma) was used to activate α7 nAChR. The perfusion medium was manually switched between ND96 with or without agonist using a Valve Driver II (General Valve Corporation, Fairfield, NJ). 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 μls) fed through a custom-made manifold mounted immediately above the oocyte. ACh pulses were applied for 2 s at 6-min intervals. After each application, the cell was superfused for 1 min with agonist-free solution, and the flow was then stopped for 5 min before agonist solution was re-introduced. Peptide was applied when responses to three consecutive agonist applications differed by less than 10%. After the 1-min agonist washout step was stopped, 5.5 μl of a 10-fold concentrated peptide solution were pipetted directly into the static bath, mixed by repeated pipetting, and incubated for 5 min prior to application of agonist. Addition of toxin directly to the recording chamber preserved material and avoided potential adhesion of the toxin to tubing surfaces. To determine estimates of potency, estimates of specific responses were fitted to the data by the equation percent response = 100/(1 + ([toxin]/IC50)α) using Prism software (GraphPad v 3.0 for Macintosh, San Diego, CA). Estimates of toxin washout kinetics, agonist responses were measured at 2-min intervals under constant superfusion. For toxin application, oocytes were superfused for 1 min before a 1-min incubation with the toxin followed by the next agonist application.

**NMR Solution Structure of GID**

**Sample Preparation—**Samples for 1H NMR measurement contained ~2.5 mM synthetic GID in 90% H2O, 10% D2O or 100% D2O at pH 2.8.

**NMR Spectroscopy—**Spectra were obtained on a Bruker DMX 750 spectrometer at 280 and 287 K. All spectra were acquired in phase-sensitive mode using TIPPI (25). The homonuclear spectra recorded included double quantum filtered DQF-COSY (26), TOCSY (27) using a MLEV-17 spin lock sequence (28). 4096 data points were fitted to the 2-D 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-dimensional and TOCSY spectra were run immediately after dissolving the sample in D2O. All spectra were processed on a Silicon Graphics workstation using XWINNMR (Bruker). The f1 dimension was zero-filled to 2048 spikes, and the 2-D spectrum was multiplied by a sine-squared function shifted by 90° prior to Fourier transformation. Processed spectra were analyzed and assigned using the program XEASY (32). Spectra were assigned using the sequential assignment protocol (33). The process was facilitated, in part,
using the automatic assignment program NOAH, which is part of the DYANA package (34).

Structure Calculations—Cross-peaks in NOESY spectra recorded in 90% H2O, 10% D2O with mixing times of 350 and 150 ms were integrated and calibrated in XEASY, and distance constraints were derived using DYANA. Backbone dihedral angle restraints were derived from 3JHNH coupling constants measured from line shape analysis of antiphase cross-peak splitting in the DQF-COSY spectrum. Angles were restrained to \(-120^\circ \pm 30^\circ\) for \(3J_{HH} > 8.5\) Hz and to \(-60^\circ \pm 30^\circ\) for \(3J_{HH} < 5\) Hz. Stereoelectronic assignments of \(\beta\)-methylene protons and \(\chi_1\) dihedral angles were derived from \(3J_{\beta\beta}\) coupling constants, measured from ECOSY spectra, in combination with NOE peak intensities (35). Slowly exchanging amide protons identified by D2O exchange experiments were used in conjunction with preliminary structures to determine hydrogen bonds. In cases where hydrogen bonds could be determined and unambiguously assigned, appropriate distance restraints were included in the subsequent calculations. Preliminary structures were calculated using a torsion angle simulated annealing protocol within DYANA. Results were refined using a torsion angle dynamic, a cooling phase with 4000 steps of 0.015 ps of torsion angle dynamics, a cooling phase with 4000 steps of 0.015 ps of torsion angle dynamics during which the temperature was 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 (39). The refinement in explicit water involves the following steps. First heating to 500 K via steps of 100 K, as described by Linge and Nilges (39). The refinement in explicit water 

RESULTS

Isolation and Chemical Characterization of GID

Isolation and Sequence of GID—In the search for new \(\alpha\)-conotoxins, the crude venom of \(C. geographus\) was screened for inhibition of agonist-evoked currents at muscle nAChR and different combinations of neuronal nAChR subunits (\(\alpha_3\beta_2\), \(\alpha_5\beta_4\), \(\alpha_4\beta_2\), \(\alpha_4\beta_4\), and \(\alpha_7\)) heterologously expressed in \(Xenopus\) oocytes. Crude venom (50 \(\mug\) protein/ml by BCA assay) caused 100% block of the \(\alpha_3\beta_2\) and \(\alpha'7\) nAChR subtypes and had weak activity at \(\alpha_4\beta_2\). LC/MS analysis of the crude venom, and MS analysis of individual fractions, confirmed the presence of the known muscle selective \(\alpha\)-conotoxins GI, GIA, and GIB, while GII and the neurally active GIC were not found (Fig. 2, A and B). Thus this pool of \(C. geographus\) crude venom appeared to contain a novel neurally active \(\alpha\)-conotoxin. Inhibitory activity at the \(\alpha_3\beta_2\) and \(\alpha'7\) subtypes was used to guide fractions of the crude venom, yielding a single component of 2184.9 Da (Fig. 2, A and B) that inhibited \(\alpha_3\beta_2\), \(\alpha'_7\), and \(\alpha_4\beta_2\) nAChR subtypes. Edman sequencing of the reduced and alkylated peptide, together with mass spectrometry evidence for the \(\gamma\)-carboxyglutamic acid (\(\gamma\)), revealed a new \(\alpha_7\)-conotoxin sequence IRD\(\gamma\)CSCNPACRVNNOHVC (Fig. 1), that we named \(\alpha\)-GID following the nomenclature proposed by Olivera and coworkers (8). The identical sequence was also identified in \(Conus tulipa\) crude venom. \(\alpha\)-GID possesses an NN(P/O) motif also found in \(\alpha\)-conotoxins EpI, PnIa, and PnIB (Fig. 1A) but additionally has a four residue N-terminal tail. GID is the first non-amidated, neurally active \(\alpha\)-conotoxin identified.

Chemical Synthesis of GID and Analogues—To characterize GID and investigate the influence of the N-terminal tail on its functional properties, GID and an analogue missing the four

---

2 L. Thomas and R. J. Lewis, unpublished data.
nal amino acids of GID reduced the activity of the peptide at the α4β2 combination by ~4-fold (Fig. 3B). Interestingly, GIDΔ1–4 (1–10 μM) caused only a partial block (~40%) of α4β2 receptors, indicating that the tail of GID was required for full antagonist activity at this subtype combination (no partial agonist activity was detected with this truncated analogue, data not shown). However, potency at the α7 and α3β2 subtypes was little influenced by removal of the four N-terminal residues. Activity of [R12A]GID at the α4β2 and α7 subtype was reduced ~10-fold compared with GID, whereas activity at the α3β2 subtype was little affected (Fig. 3, B–D). An analogue in which the four N-terminal amino acid residues of [R12A]GID were removed ([R12A]GIDΔ1–4) did not cause any inhibition of the α4β2 receptor at 3 μM (data not shown).

Influence of the N-terminal Sequence on the Off-rate Kinetics—Inhibition of the α4β2 nAChR by GID and its analogues was rapidly reversible, with at least 90% recovery of responses seen after 2 min (data not shown). However, recovery of the α3β2 and α7 subtypes from GID block was significantly slower, requiring 10 and 15 min, respectively (Fig. 4, A and C). In contrast, inhibition of both α3β2 and α7 receptors by GIDΔ1–4 was reversed after a ~2-min washout (Fig. 4, B and D). Recovery from block by [A10L]PnIA, which does not have an N-terminal tail, was rapid at the α3β2 subtype but slow at the α7 subtype (data not shown).

Structural Characterization of GID

NMR Assignments—All spectral data on GID were recorded in either 90% H2O, 10% D2O or 100% D2O. Spectra recorded at 287 K were primarily used for assignments, while spectra measured at other temperatures were used to resolve ambiguities.

Structure Determination and Analysis—Analysis of the 350-ms NOESY spectrum (750 MHz, 287 K) using the program XEASY allowed the assignment of each spin system to a specific amino acid. All non-intrarresidual peaks were subsequently assigned both manually and using the NOAH automatic assignment within the DYANA program package. 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. A set of restraints consisting of 183 NOE-derived distances and 15 dihedral angle restraints was used in the final calculations. These restraints included 82 sequential, 35 medium range, 10 long range, and 56 intraresidue distances, 6 φ angle restraints (Cys5, Cys6, Arg12, Val13, His17, and Cys19), and 9 χ1 angle restraints (Cys5, Cys6, Asn8, Cys10, Val13, Asn14, His17, Val18, and Cys19). Side-chain angle restraints were derived on the basis of coupling constants and NOE intensities from a 150 ms NOESY spectrum. There were also four restraints included for two hydrogen bonds identified in preliminary structures. In the final round of structure calculations, these restraints were used to calculate a family of 50 structures, using a torsion angle simulated annealing (37, 38) protocol within CNS version 1.0 (36). Structures consistent with the restraints were subjected to further molecular dynam-ics and energy minimization in a water shell.

Secondary Structure—The differences between the Hα chemical shifts of GID and random coil values (44) are shown in Fig. 5. A negative secondary shift (upfield) for several residues indicates that GID contains helical structural elements. The longest uninterrupted region of negative secondary shifts is

---

**Table I.** Isolation and Structure-Activity Studies of α-GID

<table>
<thead>
<tr>
<th>α-Conotoxin</th>
<th>α3β2</th>
<th>α4β2</th>
<th>α7</th>
</tr>
</thead>
<tbody>
<tr>
<td>GID</td>
<td>3.1  (1.16)</td>
<td>152 (1.13)</td>
<td>4.5 (1.06)</td>
</tr>
<tr>
<td>GIDΔ1–4</td>
<td>4.6  (1.07)</td>
<td>670 (1.16)</td>
<td>5.5 (1.15)</td>
</tr>
<tr>
<td>[R12A]GID</td>
<td>10 (1.14)</td>
<td>2000 (0.86)</td>
<td>48 (1.26)</td>
</tr>
</tbody>
</table>

---

The differences between the Hα chemical shifts of GID and random coil values (44) are shown in Fig. 5. A negative secondary shift (upfield) for several residues indicates that GID contains helical structural elements. The longest uninterrupted region of negative secondary shifts is...
function from DYANA significantly higher than observed for the globular conformation. The beads and ribbon conformations had target functions of 17.45 and 13.44, respectively, compared with 0.16 for the globular conformation. Structures were also calculated without S–S restraints, and the average target function was 6.94. Analysis of the S–S distances in these structures revealed Cys$^{5}$–Cys$^{11}$ was the most likely connectivity. The next most likely bond was between Cys$^{6}$–Cys$^{10}$; however, the distance between Cys$^{5}$–Cys$^{19}$ was not significantly different. In summary, the structure calculations with and without the disulfide bonds confirmed that GID adopted the globular conformation, and thus had the same disulfide bond pairings as other α-conotoxins.

**Description of the Three Dimensional Structure of GID**—A family of the 20 lowest NOE energy structures was chosen from the final set of 50 structures to represent the solution structure of GID. The statistics for these 20 structures, which had no distance violations greater than 0.2 Å and no dihedral angle violations greater than 3.0°, are given in Table II. The structures were well defined with the exception of the N-terminal tail, which had few experimental restraints defining this region. The mean RMSD over residues 4–19 was 0.34 ± 0.17 Å for the backbone atoms and 1.43 ± 0.32 Å for the heavy atoms. The family of structures obtained superimposed over the backbone atoms of residues 4–19 is shown in Fig. 6A. The ribbon representation of the lowest energy structure is shown in Fig. 6B. Analysis of the backbone angles reveals that 85% of the residues lie in the most favorable regions of the Ramachandran plot, 14% are in the additionally allowed region, 0.5% in the generously allowed region, and 0.5% in the disallowed region. The residues in the generously allowed and the disallowed regions correspond to Asp$^{3}$ and Ile$^{1}$, respectively, both in the poorly defined N-terminal tail.

The solution structure of GID was determined to be a highly compact globular structure consisting of a central region of α-helix and β-turns at both the N and C termini. The α-helix comprises residues 9–13 and has two turns. H-bonds between Asn$^{3}$–Cys$^{11}$ (HN) and Pro$^{5}$–Val$^{13}$ (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 1–5 may be associated with flexibility of this region in solution. Analysis with PROMOTIF reveals that residues 5–8 are involved in a type I β-turn, while residues 15–18 are involved in a type II β-turn in 7 of 20 final structures.

A surface representation of GID illustrates a distinct hydrophobic face (Fig. 7A) comprising residues Pro$^{5}$, Ala$^{10}$, Val$^{13}$, Hyp$^{16}$, and Val$^{17}$. The hydrophilic residues on the surface include Ser$^{2}$, Asn$^{5}$, Asn$^{14}$, and Asn$^{15}$. There are also four charged residues on the surface of the molecule. Three (Arg$^{2}$, Asp$^{3}$, and Gla$^{4}$) are part of the disordered N-terminal
termini are labeled. The coordinates for /H9251
bic face.

3142

with the Research Collaboratory for Structural Bioinformatics Protein –
between residues 4

20 GID structures superimposed over the backbone atoms of residues

Blabeled.

C. geographus

pool of

nAChRs for prey capture. GID appears to replace GIC in this

/H9251

previously described

pre-pro regions of

/H9251

age site is not utilized in GID, it appears that the γ-carboxy-
molecule superimposed over the α-helical residues. Negatively and

/B

moiety 

Pro6, Pro7, Ala10, and Pro13 in GID.

hydrphobic residues are in gray. Cysteine residues involved in disulfide

sites contain Arg-Arg, Lys-Lys, Gln-Arg, Lys-Arg, or Arg-

Asp motifs as the cleavage site. For example, ImIIA utilizes

Ile1, Arg2, Asp3, Gla4) including the post-translationally modiﬁed γ-carboxyglutamic acid (Gla). An examina-

of the pre-pro regions of α-conotoxins from the SWISS-

Ile-Arg-Asp-Tyr (accession number Q9U619) to leave a Tyr at

position 1 in the mature conopeptide. Since the Arg-Asp cleav-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-

age site is not utilized in GID, it appears that the γ-carboxy-
cytes (10, 45, 46, 47) (Fig. 1). Other α-conotoxins such as PnIA and PnIB inhibit both the α7 and α3β2 subtypes with nanomolar potencies, with PnIA being α3β2 selective and PnIB being α7 selective (42, 43). GID also has low nanomolar potency at these two nAChR subtypes, but additionally targets the α4β2 receptor at nanomolar levels. Thus GID has structural features that allow it to recognize a broader range of nAChR binding sites than other α-conotoxins, making it a versatile tool for structure-activity studies at the ACh binding site. Comparison of its structure with those of more “specialized” α-conotoxins may reveal the critical determinants for subtype selectivity.

The ACh binding site has been localized at the interface of an α-subunit (+ face) and the respective non-α subunit (− face – in case of the α7 nAChRs) (48–50). To date, there is limited information on the binding mode of neuronalective α-conotoxins and the factors that determine subtype selectivity. In a study to determine the direct interactions between amino acids of the α7 receptor and α-conotoxin ImI, Quiram et al. (49) found that the two exposed loops of the peptide interact with different subunits of the α7 receptor, thus bridging the interface between two adjacent subunits. However, a similar mode of binding was not found for PnIB (51). Instead, the key bioactive residues of this peptide form a localized hydrophobic patch that appears to interact mostly with the + face of the α7 binding site. Various neuronalective α-conotoxins might have evolved that have one or more attachment points and target different microdomains that overlap the ACh binding site on nAChRs (51). Therefore, it might be useful to further subgroup the neuronalective α-conotoxins based on their subunit specificity and sequence similarity in order to compare structures that are likely to have similar binding modes.

The three-dimensional solution structure of α-conotoxin GID reveals that it is a well-defined molecule, with a backbone RMSD over residues 4–19 of 0.34 Å; however, residues 1–3 are completely disordered. The side-chains are well defined, with the exception of Arg2, Gla4, and Arg12. The core of the molecule is occupied by the cysteine residue side-chains, whereas the side-chains of the remaining residues are solvent-exposed. The NMR structure of GID was compared with the crystal structure of PnIA (17), an α-conotoxin with the same global fold and related activity at α7 and α3β2 (Fig. 7B). Superimposition of the α-helical backbone residues of GID with PnIA gives an RMS deviation of 0.29 Å. This similarity is evidence that α4β7-conotoxins utilize the same highly conserved, well-defined backbone structure to present a range of different side-chains for interaction with specific nAChRs.

Charge distribution appears to be an important factor that determines nAChR selectivity, with neuronal specific α-conotoxins being neutral or negatively charged, and muscle-specific α-conotoxins having a net positive charge (52). For example, PnIA contains a single negatively charged residue, Asp14, which together with a positive N terminus and neutral, amidated C terminus results in a net charge of zero. At physiological pH, GID has four charged residues including two positive (Arg2 and Arg12) and two negatively charged residues (Asp3 and Gla4). Combined with balancing positive and negatively charged termini (unlike most other conotoxins GID contains no amidated C terminus) the net charge is close to zero. A comparison of the surfaces of GID and PnIA shows that both molecules have a similar hydrophobic patch on one face of the molecule. This patch includes amino acid residues Pro6, Pro7, Ala10, and Pro13 in PnIA (Fig. 7B), and the corresponding residues Pro9, Ala10, Val13, and O16 in GID (Fig. 7A). A similar cluster of hydrophobic residues (Leu5, Pro6, Pro7, Ala9, and Leu13) has been shown to be important for the high affinity binding of PnIB to the α7 receptor (51). Exchanging Ala with Leu at position 10 in PnIA ([A10L]PnIA) enhanced α7 and reduced α3β2 potency, suggesting that a long aliphatic side-chain in this position favored α7 selectivity (42, 43). In support, the α4β7-conotoxin GIC has a Gly in position 10 and is a potent α3β2 inhibitor (45) (see Table III). GID, which has similar potency at both α7 and α3β2 receptors, has an intermediate length side-chain (Val13) in the equivalent position to Ala and Leu. Taken together, it appears that the length of the side-chain in position 10 (or position 13 in GID) of these α4β7-conotoxins correlates with their selectivity for α7 or α3β2 receptors: the longer the aliphatic side-chain the more α7-selective and less α3β2-selective the peptides become (Table III).

The neighboring amino acid residues in positions 9 and 11 also seem to contribute to this selectivity: Arg12 of GID (equivalent to Ala9 of PnIA), contributes to α7 selectivity, since replacing it with an Ala reduced α7 activity by 10-fold without affecting activity at the α3β2 receptor. In contrast, Asn11 may contribute more to α3β2 versus α7 selectivity, since [N11S]PnIA was 24-fold less active at the α3β2 combination but only 7-fold less active at the α7 subtype as compared with PnIA. Likewise, PnIB, which has a Ser in position 11, was ~20-fold less active at α3β2 receptors but only 5-fold less active at the α7 nAChR than [A10L]PnIA (43). Activity at α7 was little altered in the Asn12Ala and Pro13Hyp analogues of PnIB (51). However, their conservation in most α4β7-conotoxins suggests they have evolved as a structurally important motif. As positions 14 and 15 in PnIB also had little influence on affinity (51), it appears that the side-chains of the first three N-terminal residues in loop II of these α4β7-conotoxins play a key role in determining potency and selectivity at α3β2 and α7 subtypes.

GID contains a unique N-terminal tail comprising four amino acids. Removal of these residues did not affect activity at α7 and α3β2 subtypes but strongly reduced block at the α4β2 subtype, turning GID into a partial inhibitor at this receptor. These results suggest that N-terminal residues outside the cysteine framework can contribute to α4β2 activity. However, this N-terminal motif is not essential for full inhibition of α4β2, since GIC is a full inhibitor of the α4β2 receptor with an IC50 value of 300 nM (45). The reduced times to washout of GID[1–4] from the α7 and α3β2 receptors suggest that the N-terminal

<table>
<thead>
<tr>
<th>α-Conotoxin</th>
<th>α3β2</th>
<th>α7</th>
<th>α3β2/α7</th>
<th>Consensus sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIC</td>
<td>9.6</td>
<td>252</td>
<td>0.04</td>
<td>CASN9Q</td>
<td>(43)</td>
</tr>
<tr>
<td>PnIA</td>
<td>10</td>
<td>48</td>
<td>0.2</td>
<td>CAANN9</td>
<td>present study</td>
</tr>
<tr>
<td>[R12A]GID</td>
<td>3.1</td>
<td>4.5</td>
<td>0.7</td>
<td>CRVNN9</td>
<td>present study</td>
</tr>
<tr>
<td>GID</td>
<td>99</td>
<td>12.6</td>
<td>7.9</td>
<td>CALKSN</td>
<td>(43)</td>
</tr>
<tr>
<td>[A10L]PnIA</td>
<td>1970</td>
<td>61</td>
<td>32</td>
<td>CALKSN</td>
<td>(43)</td>
</tr>
</tbody>
</table>
tail can stabilize the binding of GID to α1 and α3β2 receptors, presumably by interacting with residues near the ACh binding pocket that are not accessible to smaller α-conotoxins. The reduced activity of [R12A]GID at the α4β2 receptor indicates that Arg23 in loop II also contributes to GID block of α4β2. Thus GID reveals novel features contributing to α-conotoxin binding to the α4β2 receptor. Improving the structural stability of the N-terminal tail in GID or adding it to other α-conotoxins provides new avenues for the development of α4β2 selective inhibitors.

Acknowledgments—We thank Trudy Bond for amino acid analysis, Alun Jones for LC/MS analysis, Roger Pearson for peptide sequencing, and Richard Clark for critical reading of the article.

REFERENCES

Isolation, Structure, and Activity of GID, a Novel α4/7-Conotoxin with an Extended N-terminal Sequence

Annette Nicke, Marion L. Loughnan, Emma L. Millard, Paul F. Alewood, David J. Adams, Norelle L. Daly, David J. Craik and Richard J. Lewis

doi: 10.1074/jbc.M210280200 originally published online November 4, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M210280200

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 52 references, 12 of which can be accessed free at
http://www.jbc.org/content/278/5/3137.full.html#ref-list-1