α-Conotoxin GIC from Conus geographus, a Novel Peptide Antagonist of Nicotinic Acetylcholine Receptors*

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Many venomous organisms produce toxins that disrupt neuromuscular communication to paralyze their prey. One common class of such toxins comprises nicotinic acetylcholine receptor antagonists (nAChRs). Thus, most toxins that act on nAChRs are targeted to the neuromuscular subtype. The toxin characterized in this report, α-conotoxin GIC, is a most striking exception. The 16-amino acid peptide was identified from a genomic DNA clone from Conus geographus. The predicted mature toxin was synthesized, and synthetic toxin was used in all studies described. α-Conotoxin GIC shows no paralytic activity in fish or mice. Furthermore, even at concentrations up to 100 μM, the peptide has no detectable effect on the human muscle nicotinic receptor subtype heterologously expressed in Xenopus oocytes. In contrast, the toxin has high affinity (IC50 ~1.1 nM) for the human α3β2 subunit combination, making it the most neuronally selective nicotinic antagonist characterized thus far. Although α-conotoxin GIC shares some sequence similarity with α-conotoxin MII, which is also a potent α3β2 nicotinic antagonist, it is much less hydrophobic, and the kinetics of channel block are substantially different. It is noteworthy that the nicotinic ligands in C. geographus venom fit an emerging pattern in venomous predators, with one nicotinic antagonist targeted to the muscle subtype (thereby causing paralysis) and a second nicotinic antagonist targeted to the α3β2 nAChR subtype (possibly inhibiting the fight-or-flight response).

Many organisms employ toxins that act on nAChRs to defend against predators or to facilitate prey capture. Numerous low molecular weight toxins, characterized from a variety of biological sources, are likely used to discourage consumption by predators. Nicotine, an alkaloid from the tobacco plant, causes paralysis when ingested by insects. Nicotine extracts have been used since the 1900s as a natural insecticide (1). The harvest of tobacco plants by humans commonly leads to a syndrome known as green tobacco sickness. This occurs when nicotine is absorbed through the skin, leading to weakness, nausea, vomiting, dizziness, abdominal cramps, headache, and difficulty breathing (2–4). d-Tubocurarine is isolated from the Chondodendron tomentosum bush. Arrows tipped with curare have been used for centuries to hunt wild game in South America (5). Death of the prey results from paralysis of skeletal muscles. Lophotoxins are isolated from Lophogorgia pseudo-pterogorgia (a species of soft coral); lophotoxins bind irreversibly to the muscle nAChR by forming a covalent bond with a tyrosine residue in the α-subunit of the receptor (6, 7). Erythrodine is an alkaloid with curare-like activity that is isolated from the seeds of the trees and shrubs of the genus Erythrina. Methyllycaconitine is a tertiary diterpenoid isolated from the seeds of Delphinium brownii (the larkspur plant). Larkspurs are widely distributed in western North America and they kill more cattle on range lands than any other poisonous plant (8). Neosurgeratoxin is the primary toxin component of the Japanese ivy mollusc, Babyllonia japonica (9).

Among toxins used by venomous organisms to capture prey, the most well known examples are the snake venom polypeptides. More than 90 neurotoxin antagonists of nAChRs have been isolated from dozens of species of land and sea snakes of the Elapidae and Hydrophiidae families. The α-neurotoxins are a dominant component of snake venoms. The most extensively characterized α-neurotoxin is α-bungarotoxin from the Taiwanese banded krait. α-Bungarotoxin causes paralysis and death in prey by binding (with near irreversibility) to the neuromuscular junction nAChR (for a review, see Ref. 10). α-Conotoxins are small disulfide-rich peptides that have been isolated from Conus. Conus is a large genus of predatory marine snails, some of which feed on fish. A major component of the complex venomous arsenal that the fish-eating Conus employ are toxins that act at the muscle nicotinic receptor subtype. Examples include the α-, αα-, and ω-conotoxins (11). In this report, we describe the cloning and synthesis of a novel α-conotoxin from the fish-eating cone snail Conus geographus. In contrast to previously isolated α-conotoxins from this species, the new α-conotoxin has no detectable activity at the muscle subtype of receptor, but instead, it potently targets neuronal nAChRs.

EXPERIMENTAL PROCEDURES

Identification and Sequencing of a Genomic DNA Clone Encoding α-Conotoxin GIC—DNA from C. geographus hepatopancreas was isolated using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Frozen tissue was placed in 600 μl of lysis buffer, homogenized with a disposable microcentrifuge pestle, and digested overnight with 60 μg of proteinase K at 55 °C. The remainder of the procedure followed the kit manufacturer’s suggested protocol for marine invertebrates.

The resulting genomic DNA was used as a template for PCR using oligonucleotides, tailed for cloning, corresponding to the 3’-end of the

This paper is available on line at http://www.jbc.org

Printed in U.S.A.

* This work was supported by National Institutes of Health Grants MH 53831 and GM 48677. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF526267.

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‡ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; Fmoc, N-(9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography.
introns preceding the toxin region of α-conotoxin prepropeptides and the 3′-UTR (untranslated region) sequence of the α prepeptide (12).

The resulting PCR product was purified using the High Pure PCR product purification kit (Roche Molecular Biochemicals) following the manufacturer’s suggested protocol. The eluted DNA fragment was annealed to pAMPl vector, and the resulting product was used to transform competent DH5α cells with the CloneAmp pAMP system for rapid cloning of amplification products (Invitrogen) following the manufacturer’s suggested protocols.

The nucleic acid sequences of the resulting clones were determined according to the standard protocol for the Sequenase version 2.0 DNA sequencing kit described previously (13).

**Chemical Synthesis—**Peptide was synthesized, 0.45 mmol/g, on a Fmoc O-amino acid resin (Applied Biosystems, catalog No. 401435) using Fmoc chemistry and standard side chain protection except on cysteine residues. Cys residues were protected in pairs with either S-trityl on Cys and Cys or S-acetamidomethyl on Cys and Cys. Amino acid derivatives were from Bachem (Torrance, CA). The peptides were removed from the resin and precipitated; a two-step oxidation protocol was used to selectively fold the peptides as described previously (14). Briefly, the first disulfide bridge was closed by dripping the peptide into an equal volume of 20 mM potassium ferricyanide, 0.1 M Tris, pH 7.5. The second disulfide bridge was closed by eluting the peptide into an equal volume of 150 mM NaCl with or without toxin using a Hamilton syringe. Fish were observed for paralysis by placing them in a 500-ml beaker of water and stirring the water with a glass rod at 120 revolutions/min. Unaffected fish were able to maintain their position in the beaker by swimming against the current. As fish became progressively paralyzed, they increasingly drifted with the current.

Young Swiss-Webster mice (~12 g) were injected intraperitoneally with 50 μl of 150 mM NaCl with and without toxin. Mice were observed for symptoms of paralysis, and death time was determined by monitoring heartbeat with a stethoscope.

**RESULTS**

Mature α-conotoxins are derived from proteolytic processing of larger precursor proteins. In the case of the α-conotoxins, this prepropeptide is ~60 amino acids, with the mature α-conotoxin sequence of 13–18 amino acids located at the C terminus of the precursor. The processing site usually consists of one or a pair of basic amino acids that immediately precedes the mature toxin in the precursor sequence (12). We exploited the conserved features of the α-conotoxin gene structure to isolate a novel α-conotoxin gene. PCR primers were designed to directly amplify the α-conotoxin gene sequence from genomic DNA. PCR amplification of genomic DNA from C. geographus yielded a single specific α-conopeptide gene product, which was cloned and sequenced. Three identical clones representing the
α-Conotoxin GIC, a Neuronal nAChR-selective Peptide

Chemical Synthesis of GIC—Solid phase synthesis of the α-conotoxin GIC

RESULTS

Bioassay—C. geographus feeds on fish by first entrapping its prey with a distensible stomach and then harpooning and envenomating the fish. C. geographus produces both α-conotoxin GI and the newly isolated α-conotoxin GIC. α-Conotoxin GI is known to act selectively at the muscle subtype of receptor (17). One nmol of GI injected intramuscularly into fish produced signs of paralysis in 60 s or less, with full paralysis by 3 min (n = 3). In contrast, 3.5 nmol of α-conotoxin GIC did not produce any signs of paralysis (n = 3).

The effects of these toxins were also assessed in young Swiss-Webster mice as described under “Experimental Procedures.” 25-day-old mice were injected intraperitoneally with 50 μl of solution containing either α-conotoxin GI or α-conotoxin GIC. Two nmol of α-conotoxin GI caused paralysis and death in less than 5 min (n = 2). In contrast, 5 nmol of α-conotoxin GIC did not produce any observable motor deficits or paralysis (n = 3).

Electrophysiology—Results obtained from the bioassays indicated that α-conotoxin GIC had little if any activity on the neuromuscular nAChR. To examine this further, we heterologously expressed the human muscle subtype of nAChR.


trometry of synthetic α-conotoxin GIC was consistent with the amidated sequence (monoisotopic MH⁺: calculated, 1609.6; observed, 1609.5).

Synthetic α-conotoxin GIC eluted considerably earlier from the reverse phase HPLC column compared with α-conotoxin MII, a peptide isolated from another fish-hunting cone (see Fig. 2). The greater hydrophilicity of GIC seemed to have practical consequences for peptide handling. Although we did not fully investigate this phenomenon, it appeared that α-conotoxin GIC did not significantly stick to the electrophysiological recording chamber as does α-conotoxin MII. Therefore, bath applications of toxin in the recording chamber gave similar or identical results to perfusion-applied toxin. This is in contrast to the more hydrophobic α-conotoxin MII, in which bath application of toxin can lead to a 5–10-fold underestimation of potency (compared with perfusion application of toxin).

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Fig. 5. Wash-out kinetics of α-conotoxins GIC and MII. A, nAChR block by α-conotoxin GIC is rapidly reversible. After control responses were obtained, 100 nM α-conotoxin GIC was applied to human α3β2-expressing oocytes for 10 min. The oocyte was then continuously perfused with buffer without toxin while responses to ACh were obtained. Similar results were obtained in two other experiments. B, the same protocol was used for application of 100 nM α-conotoxin MII. The recovery from block was ~20-fold slower than in the case of α-conotoxin GIC.

Table II

<table>
<thead>
<tr>
<th>α-Conotoxin</th>
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<th>Sequence</th>
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<td>Neuronal</td>
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<td>NGCCCHPACARKYNIC#</td>
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shown in Fig. 5, block by kinetics of block by these toxins are substantially different. As shown in Fig. 5, block by a-conotoxin GIC is reversed relatively rapidly (>50% recovery in 1 min) upon toxin washout. In contrast, block by a-conotoxin MII is only slowly reversed, with ~50% recovery after 19 min.

**DISCUSSION**

We have characterized a 16-amino acid conotoxin with four Cys residues. The homology of the encoding gene with other conotoxin genes reveals that the peptide belongs to the A-superfamily of *Conus* toxins. The A-superfamily comprises a variety of peptides, most of which act on nAChRs, although others act at potassium or sodium channels (18). The sequence and activity of the newly characterized toxin relegates it to the a-conotoxin family, members of which act on nAChRs.

The total chemical synthesis of the new peptide, a-conotoxin GIC, was carried out using orthogonal protection of Cys residues to direct disulfide bond formation in the previously characterized pattern of a-conotoxins. We note that a-conotoxin GIC has not yet been isolated from venom. It is possible that there are post-translational modifications present in the native peptide that could influence the properties reported for the synthetic peptide described in this report.

Previously isolated a-conotoxins from the fish-hunting species *C. geographus* (see Table II) have what is referred to in the literature as a 3/5 spacing, indicating that there are respec- tively three and five amino acids in between Cys residues in the two loops of the toxin. The newly characterized toxin has a 4/7 spacing. The a/5 toxins, isolated based on their ability to cause paralysis in mice, previously have been shown to potently inhib- it the muscle nicotinic receptor subtype. Likewise, the major a/5 conotoxin from *C. geographus*, a-conotoxin GI, is a potent paralytic in fish. In contrast, a-conotoxin GIC fails to produce paralysis in either mouse or fish. Electrophysiological testing of a-conotoxin GIC on the human muscle subtype of receptor expressed in *Xenopus* oocytes is consistent with lack of para- lytic activity. Little or no block of ACh-induced current was produced at concentrations up to 100 μM toxin.

On the other hand, a-conotoxin GIC potently blocks the a/3β2 subunit combination of receptor with an IC_{50} of ~1 nM (Fig. 3 and Table I). It also has a modest amount of activity at a/3/4 and a/4β2 receptor subtypes. With respect to its pharmacological profile of activity, a-conotoxin GIC is similar to a-conotoxin MII, which also has high affinity for a/3β2 receptors, and is structurally similar in that both have an a/47 spacing. The two a-conotoxins differ in a number of respects, however. Although a-conotoxins MII and GIC share six of the eight N-terminal amino acids, they differ in seven of the eight C-terminal amino acids (Table II). a-Conotoxin GIC is much more hydrophilic than a-conotoxin MII (Fig. 2). Particularly striking is the difference in the kinetics of block between the two toxins. We previously demonstrated that block of rat a/3β2 receptors by a-conotoxin MII is only slowly reversible (t_{1/2} ~7.7 min; (19)). In this report, we demonstrate that the off-time of a-conotoxin MII is even slower at human a/3β2 receptors (t_{1/2} ~19 min). In contrast, block by a-conotoxin GIC is much more rapidly re-

versed (~20-fold faster; Fig. 5), making a-conotoxin GIC a potentially useful ligand for electrophysiological experiments, in which high potency and rapid reversibility are desired characteristics.

a-Conotoxin GIC has the highest known selectivity for neu- ronal (e.g. the a/3β2 subtype) versus muscle subtype of any nicotinic ligand characterized thus far. Although a-conotoxin MII itself is already quite selective (>1,000-fold selectivity versus the muscle subtype), a-conotoxin GIC has >100,000-fold selectivity for a/3β2 versus the muscle receptor.

Why does *C. geographus*, a mollusc that depends on paralyzing its prey, produce a nonparalytic toxin targeted to neu- ronal nicotinic acetylcholine receptors? The present findings do not answer this question, but it is worth noting compounds with similar pharmacological specificity reported from other venomous organisms. Although the major component in *Bungarus multicinctus* venom is a-bungarotoxin, this snake also produces a minor component known as k-bungarotoxin, which potently blocks a/3β2 receptors expressed in *Xenopus* oocytes (IC_{50} ~1 nM). The mechanism of action is consistent with competitive blockade (20, 21). Additional subtypes of nicotinic receptors are also blocked by k-bungarotoxin, including the muscle subtype. The kinetics of block, however, are significantly different, and an a/3β2-like receptor seems to be the primary target (20, 22).

*C. magus* produces peptides that are highly selective for the muscle subtype of nicotinic receptor and that are relatively inactive on the a/3β2 nAChRs (e.g. a-conotoxin MII) (17, 23). However, *C. magus* also produces a-conotoxin MII, which has only very weak activity at the muscle subtype (19). We establish in this report that *C. geographus* produces, in addition to its muscle-targeted toxins, a-conotoxin GIC, which has potent neuronal activity and a striking lack of activity on the muscle receptor.

Perhaps it is not coincidental that these polypeptides, k-bun-
garotoxin, a-conotoxin MII, and a-conotoxin GIC, have weak or no activity on the muscle receptor, and yet each has high affinity for a/3β2-like receptors. It seems striking that two very distinct predatory venomous organisms (snakes and snails) appear to have independently evolved not only toxins that target neuromuscular nAChRs but also those that specifically target a/3β2-like receptors. The biological utility of the latter type of toxins is unknown; however, a/3β2-like receptors exist in autonomic ganglia, where they in part modulate the fight-or-flight response (24). One possibility is that the function of the toxins that target a/3β2-like receptors is to suppress this re-

**Acknowledgment**—Alexandria Vyzavokina assisted with the synthesis of a-conotoxin GIC.

**REFERENCES**

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doi: 10.1074/jbc.M205102200 originally published online July 11, 2002

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