Contulakin-G, an O-Glycosylated Invertebrate Neurotensin*

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We have purified contulakin-G, a 16-amino acid O-linked glycopeptide (pGlu-Ser-Glu-Glu-Gly-Gly-Ser-Glu-D-GalNAc-Thr-Lys-Lys-Pro-Tyr-Ile-Leu-OH, pGlu is pyroglutamate) from Conus geographus venom. The major glycosylated form of contulakin-G was found to incorporate the disaccharide \( \beta\)-D-Gal-(1→3)-\( \alpha\)-D-GalpNAc-(1→) attached to Thr\(^{10} \). The C-terminal sequence of contulakin-G shows a high degree of similarity to the neurotensin family of peptides. Synthetic peptide replicates of Gal(\( \beta\)-D)-GalpNAc(\( \alpha\)-Thr)\(^{10} \) contulakin-G and its nonglycosylated analog were prepared using an Fmoc (9-fluorenylmethoxycarbonyl) protected solid phase synthesis strategy. The synthetic glycosylated contulakin-G, when administered intracerebroventricular into mice, was found to result in motor control-associated dysfunction observed for the native peptide. Contulakin-G was found to be active at 10-fold lower doses than the nonglycosylated Thr\(^{10} \) contulakin-G analog. The binding affinities of contulakin-G and the nonglycosylated Thr\(^{10} \) contulakin-G for a number of neurotransin receptor types including the human neurotensin type 1 receptor (hNTR1), the rat neurotensin type 1 and type 2 receptors, and the mouse neurotensin type 3 receptor were determined. The binding affinity of the nonglycosylated Thr\(^{10} \) contulakin-G was approximately an order of magnitude lower than that of neurotensin \( \text{1}_{1-13} \) for all the receptor types tested. In contrast, the glycosylated form of contulakin-G exhibited significantly weaker binding affinity for all of the receptors tested. However, both contulakin-G and nonglycosylated Thr\(^{10} \) contulakin-G were found to be potent agonists of rat neuropeptide receptor type 1. Based on these results, we conclude that O-linked glycosylation appears to be a highly unusual strategy for increasing the efficacy of toxins directed against neurotransmitter receptors.

The 500 species of predatory cone snails have evolved extremely complex venoms. Each individual species typically has at least 100 different pharmacologically active components of varying molecular weights in its venom; most are small peptides (<30 amino acids) specifically targeted to receptors or ion channels. These small, biologically active peptides found in Conus venoms can be divided into two general classes, those with sequences that contain few or no cysteine residues and those that are cysteine-rich (conotoxins). Among those in the first group, we have identified conantokinGs with no disulfide bridges (1, 2), whereas conopressins (3), contryphans (4, 5), and the bromoheptapeptide (6) all contain one disulfide bridge. The Conus peptides rich in disulfide bridges (7, 8) are synthesized from only a few conotoxin superfamilies with their diversity generated by hypermutation.

Compared with other gene translation products, Conus peptides are unusually enriched in a variety of posttranslational modifications. Some of these are widely distributed among Conus peptides (such as hydroxylation of proline to 4-trans-hydroxyproline or the amidation of the C terminus by conversion of the C-terminal glycine residue). Others appear more highly specialized (\( \gamma\)-carboxylation of glutamate to \( \gamma\)-carboxyglutamate, bromination of tryptophan to 6-bromotryptophan, sulfation of tyrosine, or the epimerization of L-tryptophan to D-tryptophan).

In this communication, we describe the purification and biochemical characterization of a novel Conus peptide that has proven to be the first known example of the neurotensin family of peptides from a nonvertebrate source. This 16-amino acid peptide, contulakin-G, contains a posttranslational-modified amino acid not previously found in a Conus peptide, an O-glycosylated threonine residue. Although evidence for O-glycosylation (at a Ser residue) was recently obtained for another Conus peptide, \( k\alpha\)-conotoxin SIVA (9), contulakin-G is the first O-glycosylated Conus peptide for which the complete structure of both polypeptide and glycan have been determined. In addition, the successful chemical synthesis of an intact, biologically active O-glycosylated gene product, as reported here, has not to our knowledge previously been achieved.

EXPERIMENTAL PROCEDURES

Crude Venom—Conus geographus specimens were collected from Marinduque island in the Philippines. The crude venom was obtained by dissection of the venom duct gland and then freeze-dried and stored at \(-70^\circ\) C.

Peptide Purification—Freeze-dried C. geographus venom (1 g) was extracted with 1.1% acetic acid and chromatographed on a Sephadex G-25 column eluted with 1.1% acetic acid as described previously (10). A peptide that makes mice sluggish and unresponsive was purified by...
A series of RP-HPLC purification on preparative and semi-preparative and analytical reverse phase C18 columns as indicated in Fig. 1. A gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid was used to elute the peptide from the columns. The major species shown in panel C was repuriﬁed further by characteristics of a single peptide, which was subjected to cleavage conditions (95% trifluoroacetic acid, 5% anisole) in 50 μl of 50 mM citrate/phosphate buffer (pH 4.5) for 53 h at 32 °C. Approximately 500 μmol of the peptide (2 μl) was incubated with 2 ml of 0.1% aqueous D-glycosidase (Diplococcus pneumoniae) (2 μl) in 50 μl of 20 mM cacodylic acid (pH 6.0) for 19 h at 32 °C.

Chemical Sequence and Amino Acid Analysis—Automated chemical sequence analysis was performed on a 477A protein sequencer (Applied Biosystems, Foster City, CA). Amino acid analysis was carried out using precolumn derivatization. Approximately 500 μmol of the contulakin-G was sealed under vacuum with concentrated HCl, hydrolyzed at 110 °C for 24 h, lyophilized, and then derivatized with p-ththalaldehyde. The derivatized amino acids were then analyzed with RP-HPLC.

Mass Spectrometry—Matrix-assisted laser desorption (MALD) (11) mass spectra were measured using a Bruker REFLEX (Bruker Daltonics, Billerica, MA) time-of-ﬂight (12) mass spectrometer fitted with a gridless reﬂectron, an N2 laser, and a 100 MHz digitizer. An accelerating voltage of +31 kV and a reﬂector voltage between 1.16 and 30 kV were employed for the post-source decay (13) measurements. The sample (in 0.1% aqueous trifluoroacetic acid) was applied with a 5 μl spotted sample using a Nanospray capillary, and analyzed. The mass accuracy was typically better than 1000 ppm for the time-of-ﬂight instrument, 200 ppm for the ion trap instrument, and 20–100 ppm for the double-focusing mass spectrometer, depending on the resolving power settings of the magnetic sector instrument employed.

Biosynthetic Protein—The solid-phase glycopeptide synthesis was carried out manually using Fmoc chemistry, with t-butyl ether side chain protection for tyrosine and serine, N-t tert-butylxoylcyxyl side chain protection for lysine, and t-butyl ester side chain protection for glutamic acid (protected amino acids were obtained from Bachem, Torrance, CA). Starting with a Wang resin, the amino acids were coupled with benzotriazoyloxyl-tris(dimethylamino)phosphonium hexafluoro phosphate/diisopropylethylamine/N-methylpyrrolidine/dichloromethane (15, 16), and the N-deprotection were done with N-methylpyrrolidone/piperidine (15, 16). The Wang resin was prepared at The Salk Bio-Rad procedure with ovalbumin as the standard.

Mass Spectrometry—Approximately 180 pmol of the peptide (6 μl) was incubated with 7.5 ml of buffer was diluted in methanol 1% acetic acid and 25% acetonitrile (1.5 ml), mixed in a thioglycerol and dithiothreitol matrix. Nano-electrospray (nano-ESI) mass spectra were measured using a Jeol HX110 (Jeol, Tokyo, Japan) double-focusing mass spectrometer, 200 ppm for the ion trap instrument, and 20–100 ppm for the Fourier transform ion cyclotron resonance mass spectrometer. Nano-electrospray (nano-ESI) mass spectra were measured using an Esquire ion trap mass spectrometer (Buker Daltonics, Billerica, MA) time-of-ﬂight (12) mass spectrometer fitted with a gridless reﬂectron, an N2 laser, and a 100 MHz digitizer. An accelerating voltage of +31 kV and a reﬂector voltage between 1.16 and 30 kV were employed for the post-source decay (13) measurements. The sample (in 0.1% aqueous trifluoroacetic acid) was applied with a 5 μl spotted sample using a Nanospray capillary, and analyzed. The mass accuracy was typically better than 1000 ppm for the time-of-ﬂight instrument, 200 ppm for the ion trap instrument, and 20–100 ppm for the double-focusing mass spectrometer, depending on the resolving power settings of the magnetic sector instrument employed.

Binding Studies—The nonglycosylated Thr19 contulakin-G and synthetic contulakin-G were assayed with the human neurotensin type 1 receptor using a Biomek 1000 robotic workstation for all pipetting steps in the radioligand binding assays as described previously (19). Competition binding assays with [3H]neurotensin1–13 (1 nM) and varying concentrations of unlabeled neurotensin1, contulakin-G, or synthetic contulakin-G were carried out with membrane preparations from HEK-293 cell line. Nonspecific binding was determined with 1 μM unlabeled neurotensin1 in assay tubes with a total volume of 1 ml. Incubation was at 20 °C for 30 min. The assay was routinely terminated by the addition of cold 0.9% NaCl (5 × 1.5 ml), followed by rapid ﬁltration through a GF/B ﬁlter strip that had been pretreated with 0.2% polyethyleneimine. Details of binding assays have been described before (20). The data were analyzed using the LIGAND program (21).

The nonglycosylated Thr19 contulakin-G and synthetic contulakin-G were separately assayed with the rat neurotensin type 1 and type 2 receptors (rNTR1 and rNTR2) and mouse neurotensin type 3 receptor (mNTR3). 125I-Tyr3 neurotensin1–13 was prepared and puriﬁed as described previously (22). The glucoprotein and glycopeptide were carried out with membrane preparations from HEK-293 cell line. Nonspeciﬁc binding was determined with 1 μM unlabeled neurotensin1 in assay tubes with a total volume of 1 ml. Incubation was at 20 °C for 30 min. The assay was routinely terminated by the addition of cold 0.9% NaCl (5 × 1.5 ml), followed by rapid ﬁltration through a GF/B ﬁlter strip that had been pretreated with 0.2% polyethyleneimine. Details of binding assays have been described before (20). The data were analyzed using the LIGAND program (21).

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Binding Experiments on Solubilized Extracts—CHAPS-solubilized extracts (100 μg) were incubated with 0.2 nm 125I-Tyr3 neurotensin1–13 for 1 h at 0 °C in 250 μl of Tris-glycine buffer containing 0.1% CHAPS. Bound ligand was separated from free ligand by filtration on GF/B filters pretreated with 0.3% polyethyleneimine. Filters were rapidly washed twice with 3 ml of ice-cold buffer and counted for radioactivity.

For binding experiments on mNTR3, membrane homogenates from mouse brain were reconstituted in 25 mM Tris-HCl buffer (pH 7.5) containing 10% (v/v) glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, 1 mM iodoacetamide, and 5 mM EDTA (Tris-glycine buffer). Membrane homogenates were pretreated by incubation at a concentration of 10 mg/ml in the Tris-glycine buffer with 0.625% CHAPS containing 0.125% cholesterol hemisuccinate (25). Solubilized extracts were recovered by centrifugation at 100,000 × g during 30 min at 4 °C and used either immediately or stored at −20 °C.

Phosphoinositides Determination—Cells expressing the rNTR1 or NTR2 were grown in 12-well plates for 15–18 h in the presence of 1 μCi

1 The abbreviations used are: RP-HPLC, reverse phase high performance liquid chromatography; Fmoc, 9-fluorenylethoxycarbonyl; Gal, galactose; GalNAc, N-acetylgalactosamine; Hex, hexose; HexNAc, N-acetyl hexosamine; icv, intracerebroventricular; LSI, liquid secondary isolation liquid Chromatography; Fmoc, 9-fluorenylethoxycarbonyl; Gal, galactose; GalNAc, N-acetylgalactosamine; Hex, hexose; HexNAc, N-acetyl hexosamine; icv, intracerebroventricular; LSI, liquid secondary isolation liquid Chromatography; MS, mass spectrometry; hNTR1, human neurotensin type 1 receptor; mNTR3, mouse neurotensin type 3 receptor; rNTR1, rat neurotensin type 1 receptor; rNTR2, rat neurotensin type 2 receptor; nNTR1, neurotensin type 1 receptor; CHAPS, 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Biotin, biotin; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; TFA, trifluoroacetic acid; glycopeptides from the glycosylated amino acids; this included chromatographic puriﬁcation on silica gel using dichloromethane ethyl acetate 4:1 as eluant and concentration and ﬁnal lyophilization of the product from benzene.
of myo-[3H]inositol (ICN) in a serum-free Ham's F-10 medium. Cells were washed with Earle buffer (pH 7.5; 25 mM HEPES, 25 mM Tris, 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 5 mM glucose) containing 0.1% bovine serum albumin and incubated for 15 min at 37 °C in 900 μl of 30 min LiCl in Earle buffer. Neurotensin-[1,16] was then added at the indicated concentrations for 15 min. The reaction was stopped by 750 μl of ice-cold 10 mM HCOOH (pH 5.5). After 30 min at 4 °C, the supernatant was collected and neutralized by 2.5 μl of 5 mM NH₄OH. Total [3H]phosphoinositides (PIs) were separated from free [3H]inositol on Dowex AG-X8 (Bio-Rad) chromatography by eluting successively with 5 μl of water and 4 μl of 40 mM and 1 μl ammonium formate (pH 5.5). The radioactivity contained in the 1 M fraction was counted after the addition of 5 μl of Ecolume (ICN).

Identification of a cDNA Clone Encoding Contulakin-G—Contulakin-G encoding clones were selected from a size-fractionated cDNA library constructed using mRNA obtained from a Conus geographus encoding clones were selected from a size-fractionated cDNA library as described previously (27). The library was screened using a probe prepared from a semipreparative C18 column and eluted with 12–42% acetonitrile in 0.1% trifluoroacetic acid (pH 2.75). The component indicated by an arrow in panel A caused wobbling and death when administered iv in mice. This was applied on a semipreparative C18 column and eluted with 12–42% acetonitrile gradient in 0.1% trifluoroacetic acid (panel B). The component marked by an arrow in panel B made mice unresponsive when administered iv. This component was further purified with an isocratic elution at 20.4% acetonitrile in 0.1% trifluoroacetic acid (panel C). A mouse injected iv with an aliquot of the component had trouble righting itself in 5 min and became very sluggish within 12 min. In approximately 25–30 min, the mouse was stretched out and lay on its stomach.

RESULTS

Purification of Contulakin-G—A fraction of C. geographus venom was detected, which made mice exceedingly sluggish (see Fig. 1). Normally, when mice that are sitting down are poked with a rod, they immediately get up and run a considerable distance. Upon iv injection of the fraction from C. geographus indicated in Fig. 1, panel A, the mice had to be poked with much more force before they got up at all, and after getting up, they would walk one or two steps and immediately sit down again. This sluggish behavior was followed through several steps of purification (panel B and C), and the apparently homogeneous peptide (panel D) was further analyzed. We have designated this peptide contulakin-G (the Filipino word tulakin’ means “has to be pushed or prodded,” from the root word tulak, to push). The “G” indicates that the peptide is from C. geographus; we have used an analogous nomenclature for other cysteine-sparse Conus peptides (i.e. conantokin-G, conpressin-G, contrypphan-R).

Biochemical Characterization of the Purified Contulakin-G—Attempted amino acid sequence analysis of the purified peptide revealed that the peptide was blocked at the N terminus. Because most N-terminal-blocked Conus peptides have a pyroglutamate residue at position 1, the peptide was treated with pyroglutamate aminopeptidase. This resulted in a shift in retention time, suggesting removal of a pyroglutamate residue. After enzyme treatment, the sequence Ser-Glu-Glu-Gly-Gly-Ser-Asn-Ala-Xaa-Lys-Lys-Pro-Tyr-Ile-Leu was obtained by standard Edman methods confirming removal of the pyroglutamate residue, where Xaa indicates no amino acid was assigned in the 9th cycle (at position 10), although a very low signal for threonine was obtained. Amino acid analyses were consistent with the presence of one threonine residue in the peptide.

To confirm the nature of the amino acid residue in position 10, a cDNA clone encoding the peptide was isolated. The nucleotide sequence and presumed amino acid sequence revealed by the clone are shown in Fig. 2. The amino acid sequence of contulakin-G obtained by direct Edman sequencing is found encoded toward the C-terminal end of the only significant open reading frame in the clone (at residues 51–66); the predicted amino acid sequence reveals that position 10 of the mature peptide (residue 60 of the precursor) is encoded by a codon for threonine. Thus, the Edman sequencing, together with cloning results, suggested that a modified threonine residue was present in position 10.

Mass spectrometric analyses (MALD, LSI, and nano-ESI) of the purified contulakin-G fraction revealed a variety of intact species as summarized in Table I. Some variation in the intensity of the different species was observed with different ionization techniques, which was ascribed to differences in the bias (29) with each ionization technique. In the following analysis, we have concentrated on the major glycoform with intact mass M₁ = 2069 observed with all of the ionization techniques investigated. The difference between the observed mass (2069 Da) and the mass calculated for the sequence assuming Thr at residue 10 (1703.83 Da) was 365 Da. Because one possible modification of threonine is O-glycosylation, we proposed, based on this mass difference, that the unidentified residue was hexose-N-acetylatedmannosamine-threonine (Hex-HexNAc-Thr), which would result in the addition of 365.13 Da. The observed masses (Table I) are consistent with the calculated monoisotopic mass of the [M₁ + H]+ or [M₁ + 2H]2+ of the proposed disaccharide-linked peptide (2069.98 or 1035.5 Da, respectively). Intense fragment ions were observed in the nano-ESI MS/MS mass spectrum of the doubly charged [M₁ + 2H]2+ intact molecule ion of contulakin-G (Fig. 3), corresponding to...
Evidence That Thr-10 is O-glycosylated—The results of the enzymatic treatment of the peptide are shown in Figs. 4 and 5. Native contulakin-G was treated with β-galactosidase isolated from bovine testes. This enzyme preferentially hydrolyzes terminal β1-3galactopyranosyl residues from the nonreducing end of glycoconjugates. After β-galactosidase treatment of the native sample, a new component was observed on RP-HPLC (see Fig. 4, inset). This component was collected and analyzed with MALD-MS in which a species was observed at m/z 1907 (Fig. 4). The difference in mass and the specificity of the enzyme are consistent with a terminal galactose residue being released. Based on the β-galactosidase hydrolysis results, we reasoned that the glycan moiety might be susceptible to O-glycosidase treatment, which liberates the disaccharide β-D-Galp-(1→3)-α-D-GalNAc bound to serine or threonine as a core unit of glycopeptides. O-Glycosidase treatment of the native contulakin-G did in fact result in a new species after the enzyme hydrolysis mixture was analyzed on RP-HPLC (see Fig. 5, inset). The new component was collected and analyzed with MALD-MS, where an m/z 1704 species was observed, consistent with loss of Hex-HexNAc (Fig. 5) (i.e., the mass was consistent with that predicted for the peptide with an unmodified threonine residue at position 10). The enzyme hydrolysis results are consistent with the presence of a β-D-Galp-(1→3)-α-

| Method of analysis | Molecule species/proposed glycan | M1 HexHexNAc | M2 SO4HexHexNAc | M3 Hex3 | M4 Hex2HexNAc2 |
|-------------------|---------------------------------|--------------|----------------|----------|----------------|
| MALD/TOF          | 2069a                           | 2186b        |                 |          |                |
| LSI/magnetic      | 2068.7                          | 2149.6       | 2436.5          |          |                |
| nano-ESI/IT       | 2068.6e                         |              |                 |          |                |
| Average values    |                                 |              |                 |          |                |
| Mono [M + H]+     | 2068.97                         | 2148.92      | 2189.94         | 2434.10  |
| Average [M + H]+  | 2070.19                         | 2150.25      | 2191.27         | 2435.53  |

a m/z 2091 and 2107, corresponding with [M1 + Na]+ and [M1 + K]+ were also observed.
b m/z 2210 and 2225, corresponding with [M3 + Na]+ and [M3 + K]+ were also observed.
c m/z 1035.3 and 1075.3, doubly charged ions were observed.
d Monoisotopic and [M + H]+ masses were calculated based on proposed glycan and contulakin-G sequence.
e Average [M + H]+ masses were calculated based on proposed glycan and contulakin-G sequence.

Fig. 2. cDNA encoding contulakin-G precursor.

Fig. 3. Nano-ESI MS/MS spectrum (m/z 1035 precursor) of native contulakin-G (286–1886 Da) (the MS/MS experiment is denoted using a suggested shorthand (37) where the closed circle represents m/z 1035 [M + 2H]+ precursor, and the arrows are directed toward the open circles which represent the fragments generated from the precursor). Above the spectrum, the structure of the glycoamino acid is represented where the arrows indicate 2 sites that lead to major fragment ions observed in the MS/MS spectrum (30).

Fig. 4. MALD mass spectra of native contulakin-G before (A) and after (B) β-galactosidase treatment. The inset shows the RP-HPLC chromatograms of native contulakin-G alone (C), β-galactosidase alone (D), and native contulakin-G (E) after β-galactosidase treatment, where the arrow indicates the component that was analyzed in panel B (in panels C thru E, the dashed lines represent the solvent gradient).

inset). The new component was collected and analyzed with MALD-MS, where an m/z 1704 species was observed, consistent with loss of Hex-HexNAc (Fig. 5) (i.e., the mass was consistent with that predicted for the peptide with an unmodified threonine residue at position 10). The enzyme hydrolysis results are consistent with the presence of a β-D-Galp-(1→3)-α-
O-Linked Glycan Mediates Invertebrate Neurotensin Activity

Synthesis of the Nonglycosylated and Glycosylated Contulakin-G—The 16-amino acid nonglycosylated peptide was chemically synthesized. The synthetic material was found to have the same retention time as the enzymatically deglycosylated contulakin-G on RP-HPLC. The 16-amino acid-glycosylated contulakin-G containing Gal(β1→3)GalNAc(α1→) attached to Thr\(^{10}\) was also synthesized. This synthetic glycosylated contulakin-G co-eluted with the native contulakin-G on RP-HPLC (see Fig. 6, panels A, B, and C). The post-source decay fragmentation spectra observed for both native and synthetic contulakin-G showed very similar fragmentation patterns (panels D and E).

Biological Potency of Synthetic Glycosylated and Nonglycosylated Contulakin-G—The loss of motor control for which the native contulakin-G was originally isolated, together with gut contraction, absence of preening/grooming, and reduced sensitivity to tail depression were signs observed when neurotensin\(_{1-13}\), nonglycosylated Thr\(^{10}\) contulakin-G, or synthetic contulakin-G were administered icv. To investigate these observations in more detail, we undertook a dose response comparison as detailed in Table II. Although the nonglycosylated Thr\(^{10}\) contulakin-G analog was active at doses of 1 nmol and higher, it was inactive at 300-pmol doses. In contrast, contulakin-G was found to elicit loss of motor control at doses of 30 pmol or approximately 5 pmol/g.

The six C-terminal amino acids of contulakin-G show significant similarity to the sequences of neurotensin\(_{1-13}\), neuropeptide (NPY), and the C terminus of xenopsin (see Table III). Because of the similar symptoms observed when either contulakin-G or neurotensin\(_{1-13}\) were administered icv and the significant homology between contulakin-G and neurotensin\(_{1-13}\), we tested the affinity of contulakin-G for a number of the cloned neurotensin receptors. As shown in Table IV, the nonglycosylated Thr\(^{10}\) contulakin-G analog was found to bind the human neurotensin type I receptor (hNTR1) with 10-fold lower affinity than neurotensin\(_{1-13}\) and even lower affinities for the other NTRs. Contulakin-G exhibited significantly lower affinity than the nonglycosylated Thr\(^{10}\) contulakin-G analog for all of the NTRs tested.

Both contulakin-G and the nonglycosylated Thr\(^{10}\) contulakin-G analog acted as agonists when tested on CHO cells expressing the rNTR1 as shown in Fig. 7. No response was observed with CHO cells expressing the rNTR2. In Fig. 7, the nonglycosylated Thr\(^{10}\) contulakin-G analog resulted in slightly lower potency (0.6 nM) but with similar efficacy as compared with neurotensin\(_{1-13}\). The synthetic glycosylated contulakin-G potency was significantly lower (20–30 nM), and the agonistic efficacy was approximately half that observed for neurotensin\(_{1-13}\).

**DISCUSSION**

*Contulakin-G and Post-translational Modification—The Co*\(\text{nus}\) peptide characterized in this report, contulakin-G, has a novel biochemical feature: a post-translationally O-glycosylated threonine not previously found in *Co*\(\text{nus}\) peptides. We demonstrated, using mass spectrometry and specific enzymatic hydrolyses, that Thr\(^{10}\) was modified with the disaccharide β-D-Galp-(1→3)-α-D-GalNAc(1→). The synthetic glycosylated and nonglycosylated forms of contulakin-G and were able to confirm the molecular structure of this major glycosylated form of the native molecule based on RP-HPLC co-elution and MS fragmentation criteria. The other more minor molecule species observed with mass spectrometry (see Table I) are not yet fully determined; their masses are consistent with glycan structural variations at peripheral sites on the characterized oligosaccharide core unit (31).

An analysis of a cDNA clone encoding contulakin-G (Fig. 2)
reveals that the prepropeptide organization of the contulakin-G precursor is similar to that of other Conus peptide precursors (8). A typical signal sequence is found, and immediately N-terminal to the contulakin-G sequence are two basic amino acids that presumably signal a proteolytic cleavage to generate the N terminus of the mature peptide (the glutamine residue would cyclize to pyroglutamate either spontaneously or because of the action of glutaminyl cyclase (32)). Although in most respects the contulakin-G precursor has the same organization as all other Conus venom peptide precursors and would be predicted to be processed in the same way, the 10 C-terminal amino acids predicted by the clone are not present in contulakin-G purified from venom. One possibility is that the clone represents a different variant; for example, one that was alternatively spliced. Alternatively, further proteolytic processing at the C terminus may be required to generate mature contulakin-G.

Over the last 20 years, an increasing number of biologically important glycopeptides and glycoproteins have been identified. Vespulakinin 1, first identified by Pisano and co-workers (33), is to our knowledge the only other O-glycosylated peptide toxin that has been isolated from venom other than Conus. Vespulakinin 1 was extracted from the venom sacs of the yellow jacket wasp, Vespula maculifrons. The peptide (TAT\*\*\*\*-RRRRPFPSPFR-OH, where the asterisk indicates an O-linked glycosylated threonine residue) contains two sequential \(\gamma\)-carboxyglutamate residues to promote \(\gamma\)-carboxyglutamate residues to promote formation of \(\alpha\)-helices (34). Conus peptides without multiple disulfides comprise a most eclectic set of families, including the conopressins, conantokins, contryphans, and now contulakin-G. The conopressins are probably endogenous molluscan peptides, clearly homologous to the vasopressin/oxytocin family of peptides; these are more widely distributed in molluscan tissues than in Conus venom ducts. However, the other non-disulfide-rich peptides (conantokins, contryphans, and contu-
O-Linked Glycan Mediates Invertebrate Neurotensin Activity

**TABLE V**

| Peptide                | Sequence                                                                  | Enzyme                  | Modification | Ref. |
|------------------------|---------------------------------------------------------------------------|-------------------------|--------------|------|
| α-Conotoxin GI         | ECDCNPACGRHYSC*                                                          | Disulfide isomerase      | Cystine      | 42   |
| μ-Conotoxin GHA        | RDCC00KKCKDRQKQR0RCACA*                                                  | Proline hydrolase        | Hyp          | 43   |
| Conantokin-G           | GRCCHPACPGBKNC*                                                          | PAM                     | −NH₂         | 44   |
| Bromocrotophyrin-R     | GECyL45L45NQ55L55R55K55SN*                                              | γ-Glutamate carboxylase  | Gla          | 45   |
| Bromoheptapeptide-Im   | GCQ0EPWC*                                                                | Bromopeptidase           | Bz-Trp       | 5    |
| α-Conotoxin Epl        | GCC55DP55RCNN55MP55Y55C*                                                | Tryptophan epimerase     | n-Trp        | 4    |
| κ-Conotoxin SIVA       | ZKSL55P55V55T55TCCG55Y55DG55TM00CRCTNSC*                                | PP GalNAc transferase +  | Glyco-Ser    | 9    |

Contulakin-G: ZSEEGGSNAS8KKPYIL PP GalNAc transferase Glyco-Thr

lakin-G) may be specialized venom peptides exhibiting unusual post-translational modifications. In addition to the O-glycosylated threonine moiety of contulakin-G described here, γ-carboxylation of glutamate residues and the post-translational epimerization and bromination of tryptophan residues were discovered in conantokins and contrphans. Table V summarizes all of the post-translational modifications found in Conus peptides to date.

**Contulakin-G, a Member of the Neurotensin Family**—Several lines of evidence are consistent with contulakin-G being the first member of the neurotensin family of peptides to be isolated from an invertebrate source. First, the C-terminal region of contulakin-G exhibits a striking degree of similarity to other members of the neurotensin family (all from vertebrates), as shown in Table IV. Furthermore, we have shown that contulakin-G competes for binding to three known neurotensin receptor subtypes; evidence that contulakin-G acts as an agonist on a cloned neurotensin receptor is also presented above. Most convincingly, however, when contulakin-G is injected into mice, the same behavioral signs are elicited as with administration of neurotensin. Thus, structural data, binding data, and in vivo behavioral symptomatology are all consistent with the assignment of contulakin-G to the neurotensin family of peptides.

Establishing that contulakin-G is an invertebrate member of the neurotensin family of peptides raises the question of whether this peptide has endogenous functions in Conus and other molluscs. Although contulakin-G may well have evolved exclusively as part of the prey capture strategy of C. geographus, we note that the only other neuropeptide homologs isolated from Conus venom, the conopressins (3), were later shown to be endogenous neuropeptides of the vasopressin family in Lymnaea, a gastropod mollusc only distantly related to Conus. Thus, the discovery of a contulakin-G makes a potential endogenous role for the neurotensin family of peptides in invertebrates worthy of further investigation.

One possible role for contulakin-G in the capture of fish prey by C. geographus is to suppress sensory circuitry in the prey. We have indicated elsewhere (8) that in contrast to fish-hunting Conus that use a “hook-and-line” strategy, C. geographus uses a “net” strategy. We suggest that instead of the exitotoxic effect of these ligands on CHO cells expressing rNTR2 using the IP accumulation assay does not correlate with the in vitro binding data; both peptides are agonists at concentrations significantly below their IC₅₀ binding affinity (524 and 79 nM, respectively). Most unexpected, therefore, given its apparently lower binding affinity, is the increased potency of glycosylated contulakin-G compared with the nonglycosylated analog after ivc administration.

Thus, the role of the glycans is somewhat paradoxical. In vitro, the glycans neither increase the binding affinity, the agonistic potency, nor agonistic efficacy. In contrast, in vivo, the glycan significantly increases the potency of the peptide. One simple explanation is that the increased potency of contulakin-G compared with the nonglycosylated analog is because of increased stability. An alternative mechanism for the increased potency is transport to the site of action facilitated by the glycans. Additionally, the glycosylated peptide may act with high affinity on as yet undefined neurotensin receptor subtype (36) or may be a selective high affinity ligand for a particular state of a neurotensin receptor subtype. Yet another possibility is that the relevant targeted neurotensin receptors may be closely co-localized with carbohydrate binding sites and that the glycan may serve as an “address label,” a mechanism postulated for certain opiate peptides.

We have begun to evaluate these different possibilities. Preliminary data supporting the increased stability hypothesis has been obtained; proteolytic degradation of contulakin-G is inhibited by the presence of the glycan moiety. The increased stability may well result in an enhanced supply of the glycopeptide at the receptor. However, the increased in vivo potency of contulakin-G conferred by O-glycosylation clearly requires a more balanced evaluation of the possibilities outlined above.

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