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The First γ-Carboxyglutamatic Acid-containing Contryphan

A SELECTIVE L-TYPE CALCIUM ION CHANNEL BLOCKER ISOLATED FROM THE VENOM OF CONUS MARMOREUS*

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Contrphans constitute a group of conopeptides that are known to contain an unusual density of post-translational modifications including tryptophan bromination, amidation of the C-terminal residue, leucine, and trytophan isomerization, and proline hydroxylation. Here we report the identification and characterization of a new member of this family, glacontryphan-M from the venom of Conus marmoreus: This is the first known example of a contryphan peptide carrying glutamyl residues that have been post-translationally carbamated to γ-carboxyglutamyl (Gla) residues. The amino acid sequence of glacontryphan-M was determined using automated Edman degradation and electrospray ionization mass spectrometry. The amino acid sequence of the peptide is: Asn-Gla-Ser-Gla-Cys-Pro-γ-Trp-His-Pro-γ-Trp-Cys. As with most other contrphans, glacontryphan-M is amidated at the C terminus and maintains the five-residue intercysteine loop. The occurrence of a γ-trytophan residue was confirmed by chemical synthesis and HPLC elution profiles. Using fluorescence spectroscopy we demonstrated that the Gla-containing peptide binds calcium with a $K_D$ of 0.63 μM. Cloning of the full-length cDNA encoding glacontryphan-M revealed that the primary translation product contains a N-terminal signal/propeptide sequence that is homologous to earlier reported contryphan signal/propeptide sequences up to 10 amino acids preceding the toxin region. Electrophysiological experiments, carried out on mouse pancreatic B-cells, showed that glacontryphan-M blocks L-type voltage-gated calcium ion channel activity in a calcium-dependent manner. Glacontryphan-M is the first contryphan reported to modulate the activity of L-type calcium ion channels.

The vitamin K-dependent γ-glutamyl carboxylase catalyzes the post-translational conversion of glutamyl residues to γ-carboxyglutamyl (Gla) residues in precursor proteins that contain the appropriate γ-carboxylation recognition site within the propeptide of the precursor (1–4). Among the classes of proteins that contain Gla, the vitamin K-dependent blood coagulation proteins have been most thoroughly studied. Upon addition of calcium they undergo a conformational transition that is a prerequisite for their interaction with biological membranes, and hence crucially important for their biological activity. Subsequent to its initial discovery, Gla was shown in 1984 to be present in venom peptides of highly specialized invertebrate systems, marine snails of the genus Conus (5, 6). It was found that a neuroactive Conus peptide, conantokin-G (17 amino acid residues) contained five residues of Gla. These discoveries indicated that the role of this post-translational modification in blood coagulation represents only a subset of Gla function in animal phyla.

The cone snail venoms contain a diverse array of paralyzing peptides (conotoxins) that are injected into prey after a cone snail harpoons its victim. The peptides specifically bind to a variety of receptors and ion channels in the neuromuscular system and interfere with their function. The Gla content varies from species to species but is especially high in the venoms of Conus textile and Conus marmoreus (7). Cloning and expression of the Conus (8–10) and Drosophila (11, 12) γ-glutamyl carboxylases has revealed a marked conservation of this gene in the animal kingdom. Experiments with crude preparations of Conus carboxylase have shown that this enzymatic reaction requires vitamin K (7, 13). Like its mammalian counterpart the Conus carboxylase requires a recognition site that resides on the propeptide of the precursor form of the toxin (14, 15). Although the γ-glutamyl carboxylases are highly conserved, the Conus and mammalian carboxylase binding sites do not bear any obvious sequence resemblance.

To date several Gla-containing conotoxins have been isolated (16–21). The metal binding properties and the three-dimensional structure of some of these conotoxins suggest a specific structural role for Gla (22–28). With the exception of conantokin-G the function of Gla in the conotoxins is, however, still unknown (5, 29, 30).

The contryphan family of conopeptides isolated from piacivo-
Glacontryphan-M, an L-type Ca\(^{2+}\) Ion Channel Antagonist

rrous (31, 32), molluscs (33), and vermiculourous (34) cone snails are distinct for their unusual density of post-translational modifications. These include bromination of tryptophan, proline hydroxylation, C-terminal amidation, and leucine and tryptophan L to D isomerization. Contrarypns cause the so-called "stiff-tail syndrome" when injected intracranially in mice (35) and body tremor and secretion of mucous substances when injected into fish (36). Recently, the first functional target for a contrypn was reported (37) as Contrypn-Vn from the wormhunting C. ventricosus was demonstrated to affect both voltage-gated and calcium-dependent K channels.

Here we describe the identification, purification, and characterization of a novel contrypn peptide, glacontryphan-M, extracted from the venom of the molluscareous C. marmoreus. Glacontryphan-M is the first example of a contrypn peptide containing Gla residues endowing it with calcium binding properties. Cloning of the cDNA of glacontryphan-M showed a high density of arginyl and lysyl residues in the propetide region compared with the prepeptide regions of the earlier described contrypns. This might indicate that these basic residues are part of the γ-carboxylation recognition site. Patch-clamp recordings carried out on insulin-secreting B-cells from the islets of Langerhans demonstrate that glacontryphan-M, in a calcium-dependent manner, specifically antagonizes L-type Ca\(^{2+}\) channel activity. Like in other neuro- and endocrine cells (38) extracellular calcium activity, specifically antagonizes L-type Ca\(^{2+}\) channels.

EXPERIMENTAL PROCEDURES

Conotoxin Purification—Frozen cone snails (C. marmoreus) were obtained from Vietnam. Lyophilized venom extract (1000 mg, from five cone snails) was dissolved in 0.2 M ammonium acetate buffer (pH 7.5) (17, 20) and chromatographed on a Sephadex G-50 superfine column (2.5 \(\times\) 92 cm) equilibrated with 0.2 M ammonium acetate buffer (pH 7.5) and eluted with a flow rate of 10.3 ml/min (Fig. 1A). The material in the major Gla-containing peak was separated on a reversed-phase HPLC column (HyChrom C18, 5 \(\mu\)m; 10 \(\times\) 250 mm) in 0.1% (v/v) trifluoroacetic acid and eluted with a linear acetonitrile gradient (a flow rate of 2 ml/min (Fig. 1B). A total of 30 nmol of Glacontryphan-M was obtained from a second reversed-phase HPLC purification step (Vydac C18 column, 5 \(\mu\)m; 46 \(\times\) 250 mm) in 0.1% trifluoroacetic acid using a linear acetonitrile gradient at a flow rate of 0.5 ml/min (Fig. 1C).

Peptide Synthesis—Peptides covering the mature toxin region of glacontryphan-M were synthesized either with a D- or L-tryptophan at position 7. The peptides were synthesized with DPTHF and D-Fmoc amino acids (Perseptive Biosystems, Framingham, MA) on a Milligen 9050 Plus peptide synthesizer (PerkinElmer Life Sciences). The peptides were deprotected and cleaved from the resin by treatment with 95% trifluoroacetic acid containing relevant scavengers. After 15 min the reactions were quenched with a fresh drop of 0.1 M ascorbic acid. Each of the oxidized peptides was applied to a reversed-phase C\(_4\) preparative column and eluted with an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid. For each of the synthesized peptides the major peak corresponding to the peptide fraction was added dropwise to an equal volume of 1 m mol iodoine in 20% acetic anhydride in 0.1% (v/v) trifluoroacetic acid. After 15 min the reactions were quenched with a fresh drop of 0.1 M ascorbic acid. Each of the oxidized peptides was applied to a reversed-phase C\(_4\) preparative column and eluted with an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid. Major peaks were analyzed with nanoelectrospray ionization mass spectrometry (NanoESI-MS). The synthesized t-contrypn-containing peptide was further purified on a reversed-phase C\(_4\) preparative column using an acetonitrile gradient supplied with 0.1% (v/v) trifluoroacetic acid, and the synthesis was confirmed by NanoESI-MS analysis and amino acid sequencing. For the synthesized t-contrypn-containing peptide, NanoESI-MS analysis revealed that only dimers had formed during the folding step. A peptide, glacontryphan-M, covering the mature toxin region of glacontryphan-M but containing Gla residues at positions 2 and 4 replacing the acidic residues was synthesized, folded, and purified according to the above procedure. The synthesis was confirmed by NanoESI-MS analysis and amino acid sequencing.

Mass Spectrometry—NanoESI mass spectra were acquired on an API QSTAR Pulsar-i quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Scie, Toronto, Canada) equipped with a Nanospray ion source (MDS Proteomics, Odense, Denmark). The samples were sprayed from a silver-coated glass EconoTip nanospray capillary supplied by New Objective (Woburn, MA). Spectra were obtained in either positive or negative ion mode. A potential of 800–1100 V was applied to the nanoflow tip in the ion source using a curtain gas (N\(_2\)) flow rate of 1.3 liters/min. The desolvation gas was heated to 500 °C. The ion spray voltage was adjusted to 4940 V with a spray voltage (S) of 1000 V. The so-called "stiff-tail syndrome" was detected by the conformation sensing of the intrinsic fluorescence was measured by addition of 0.3 M iodine in 20% acetonitrile in 0.1% (v/v) trifluoroacetic acid, followed by incubation at 50 °C for 2 h, after which iodoacetic acid was added to a final concentration of 1% to quench the reaction. The sample was then dialyzed against 3.5 M acetic acid, and the peptide was purified by reversed-phase HPLC.

Calcium Binding Measurements—Tryptophan fluorescence was measured at 20 °C in a Spex Fluoromax-3 spectrophotofluorometer (Jobin Yvon-Spex, Instruments s.a., Inc.) equipped with a 150 watt Xenon lamp. The peptide concentrations were 2 \(\mu\)M, and the solvent was metal-free 50 mM Tris-HCl, pH 7.5 containing 0.1 m NaCl. The samples were subjected to exciting light for a minimum of time to avoid photodegradation of the excitation wavelength 295 and 425 nm with an excitation wavelength of 290 nm and excitation and emission bandwidths of 2 and 8 nm, respectively. The Ca\(^{2+}\) dependence of the intrinsic fluorescence was measured by addition of 0.3–1 \(\mu\)M of Ca\(^{2+}\) to various solution conditions.
and by averaging 30 signals readings of 0.25 s each. The excitation wavelength was 290 nm and the emission wavelength 355.5 nm (corresponding to the maximum fluorescence emission intensity of the peptides). The excitation and emission bandwidths were 2 and 8 nm, respectively. The data were expressed as 1 - F/Fo, where F and Fo are the fluorescence intensities in the presence and absence of Ca2+ ions, respectively. The dissociation constant was determined by non-linear regression to fit the data to the equilibrium binding equation (43).

**Electrophysiology**—Isolated pancreatic islets from NMRI mice were dispersed into single cells using a Ca2+-free solution as described elsewhere (44). The insulin-secreting B-cells were identified by their absence of Na+ current at physiological membrane-potentials (45). Patch-clamp electrodes were made from borosilicate glass capillaries coated with polyacrylamide and fire-polished. The pipette resistance ranged between 3 and 6 MΩ when filled with pipette solution as specified below. Whole cell currents were recorded using an EPC-9 patch clamp amplifier and the software Pulse ver 8.31 (Heka Elektronik, Germany). All experiments were conducted using the perforated patch whole cell configuration in which the cytoplasm retains intact.

The standard extracellular solution consisted of 118 mM NaCl, 20 mM tetraethyl-ammonium chloride (TEA-Cl), to block voltage-gated K+ currents, 5.6 mM KCl, 2.6 mM CaCl2, 1.2 mM MgCl2, 5 mM glucose, and 5 mM HEPES (pH 7.4 using NaOH). In the Ca2+-free extracellular solution CaCl2 was substituted with 11.4 mM sucrose (to retain similar osmolarity) and 2 mM EGTA (to bind Ca2+). Glacontryphan-M, glucontryphan-M, and isradipine were added to the extracellular solution as indicated in the figures. The pipette solution contained 76 mM Cs2SO4, 10 mM NaCl, 10 mM KCl, 1 mM MgCl2, 5 mM HEPES (pH 7.35 using CsOH). Electrical contact was established by addition of the pore-forming compound amphoterican B to the pipette solution (final concentration 0.24 mg/ml; Ref. 46). Data are presented as mean values ± S.E., and statistical significance was evaluated using the Student’s t test.

**RESULTS**

**Purification and Characterization of Glacontryphan-M**—Crude C. marmoratus venom was extracted and chromatographed on a Sephadex G-50 superfine column (Fig. 1A). Every third collected fraction was analyzed for Gla content after alkaline hydrolysis. From the major Gla-containing peak, glacontryphan-M was purified by a two-step reversed phase HPLC procedure (Fig. 1, B and C). The primary sequence for the native form of the peptide was determined by automated Edman degradation to be Asn-Xxx-Ser-Xxx-Xxx-Pro-Trp-His-Pro-Trp-Xxx. Sequencing demonstrated partial deamidation (8–11%) of the N-terminal Asn residue. The presence of cysteines at positions 5 and 11 was deduced by sequencing the carboxymethylated peptide. At positions 2 and 4, γ-carboxyglutamyl acid residues were identified in a peptide that had been methyl-esterified prior to sequencing. The amino acid composition of the peptide analyzed after acid or alkaline hydrolysis was consistent with the following sequence: Asn-Gla-Ser-Gla-Cys-Gla.

**Analysis of Glacontryphan-M by NanoESI-MS—**The native form of glacontryphan-M was analyzed by NanoESI-MS in negative and positive ion modes. The mass spectrum acquired in negative ion mode is shown in Fig. 2A (m/z 1400–1480) and B (m/z 680–750). The signal observed at m/z 1470.47 Da (M-1H, Fig. 2A) corresponds to the molecular ion of the peptide and the major ion peak at m/z 734.74 Da (Fig. 2B), most likely contain the doubly (M-2H) charged ion of glacontryphan-M. The unusual isotope distribution with the second peak being more intense than the first (Fig. 2, A and B) is in all likelihood caused by the deamidation of the N-terminal Asn residue. The presence of two Gla residues in the sequence is notable in the mass spectrum via decarboxylation of the peptide resulting in a loss of 44 Da for each Gla residue (Fig. 2B). This loss of CO2 is observed at m/z 712.74 Da and 690.75 Da for the doubly charged ion. The monoisotopic molecular mass of native glacontryphan-M was determined from the negative ion mode NanoESI-MS experiments to be 1471.47 Da. These data fit well with the theoretically calculated monoisotopic molecular mass (1471.48 Da) of the peptide, assuming disulfide bonding of the cysteine residues and, like most conopeptides, amidation of the C-terminal carboxyl group (Fig. 2C). The observed molecular mass of native glacontryphan-M was further confirmed by NanoESI-MS experiments performed in the positive ion mode (not shown).

Reduction and alkylation of cysteines in glacontryphan-M with iodoacetic acid resulted in a mass increase of 118.13 Da (theoretical 118.01 Da) as compared with the native compound, consistent with two cysteine residues in the sequence (the monoisotopic molecular mass was determined to be 1589.60 Da; Fig. 3A). The S-carboxymethylated peptide was analyzed by MS/MS spectrometry in the positive ion mode. The fragment ion spectrum obtained for the selected trapped ion at m/z
TABLE I
Comparison of mature contryphan sequences

| Peptide            | Sequence       | Species         | Ref. |
|--------------------|----------------|-----------------|------|
| Des(Gly)contryphan-R | COGEFWPC-NH₂  | C. radiatus     | 35   |
| Contryphan-R       | GGOFWPC-NH₂   | C. radiatus     | 35   |
| Bromocontryphan-R  | GGOFWPC-NH₂   | C. radiatus     | 35   |
| Contryphan-Sm      | GGOFWPC-NH₂   | C. stercusmuscum| 32   |
| Contryphan-P       | GGOFWPC-NH₂   | C. purpurascens | 32   |
| Contryphan-R/Tx    | GGOFWPC-NH₂   | C. textile      | 33   |
| Contryphan-Tx      | GGOFWPC-NH₂   | C. textile      | 33   |
| Contryphan-Vn      | GDCAFWPC-NH₂  | C. ventricosus  | 34   |
| Leu-contryphan-P   | CVLFWPC-OH    | C. purpurascens | 36   |
| Leu-contryphan-Tx  | CVLFWPC-NH₂   | C. textile      | 33   |
| Glacontryphan-M    | NYSACPFWPC-NH₂| C. marmoreus    | This work |
|                    |                |                 |      |

* O, 4-trans-hydroxyproline; W, D-tryptophan; L, D-leucine; X, bromotryptophan; γ, gamma-carboxyglutamic acid.

795.80 Da (Fig. 3B) revealed b and y ions in agreement with the proposed sequence. The mass of the y1 ion further confirms that glacontryphan-M contains an amidated C terminus. The isotope pattern of the b ions was consistent with partial deamidation of the N-terminal Asn residue (Fig. 3B). The y ions gave no indication of deamidation of the C-terminal amide.

**Glacontryphan-M Contains a D-Tryptophan**—The sequence similarity of the C. marmoreus contryphan with the previously identified contryphans suggested the presence of a D-tryptophan residue at position 7 (Table I). Therefore, a synthetic peptide covering the sequence of glacontryphan-M with a D-tryptophan at position 7 and having the C-terminal carboxyl group amidated was prepared and folded. Analysis of the synthetic material by automated Edman degradation and NanoESI-MS confirmed the sequence of the peptide. Moreover the isotope pattern gave no evidence for deamidation at the N-terminal Asn residue. The synthetic contryphan was analyzed using reversed phase HPLC. Comparison of the HPLC elution profiles of the natural and synthetic peptides (Fig. 4, A and B), obtained under the same conditions, revealed the presence of D-tryptophan in the native C. marmoreus contryphan. When co-injected, the synthetic peptide and the biological native peptide co-migrated (Fig. 4C). Attempts to generate a synthetic peptide containing two L-isomers of tryptophan failed due to the exclusive formation of dimeric peptides in the folding procedure.

**Calcium Binding Properties of Glacontryphan-M**—The Gla residues in the sequence of glacontryphan-M could indicate a calcium-binding peptide. The peptide contains two tryptophan residues and we therefore used fluorescence spectroscopy to monitor Ca²⁺ binding of glacontryphan-M. Calcium binding was found to generate a 76% reduction of the intrinsic tryptophan fluorescence (Fig. 5A). The addition of Ca²⁺ to a synthetic peptide having the Gla residues replaced with Glu residues (glucontryphan-M) did not result in the same dramatic quenching which clearly indicates that the Gla residues are involved in Ca²⁺ binding (Fig. 5B). The changes in fluorescence emission as a function of Ca²⁺ concentration for glacontryphan-M were adequately described by a one-site binding equation giving an apparent K_d of 0.63 mM (Fig. 5C). In the accompanying report (47) the structure of glacontryphan-M in the absence and presence of calcium ions was determined using two-dimensional 1H NMR spectroscopy (47). A comparison of the two structures revealed subtle calcium-induced perturbations that reorder and reposition the Gla-containing N-terminal stretch of residues. The observed calcium-induced fluorescence quench of glacontryphan-M seems thus to be the result of minor localized structural perturbations and not a major conformational transition.

**Cloning of Glacontryphan-M**—The entire amino acid sequence of the precursor form of glacontryphan-M was predicted by cloning of the full-length cDNA from a C. marmoreus eDNA library. The cDNA sequence predicts a primary translation product of 63 amino acids that corresponds to the 11 residues of...
FIG. 3. Positive-ion ESI-MS and MS/MS spectra of S-carboxymethylated glacontryphan-M. A, monoisotopic molecular mass of the S-carboxymethylated glacontryphan-M was determined from the mass spectrum to be 1589.60. B, ESI MS/MS product ion spectrum of the doubly charged ion at m/z 795.80. The b and y ions according to the proposed sequence are shown. The b8 and y9 ions have been expanded to show the unusual isotope distribution observed in the b ions but not in the y ions caused by partial deamidation of the N-terminal Asn residue.
Glacontryphan-M, an L-type Ca\textsuperscript{2+} Ion Channel Antagonist

currents were measured on single pancreatic B-cells using the perforated patch configuration were performed. To elicit Ca\textsuperscript{2+} currents 100-ma depolarizations from −70 to 0 mV were applied to the cell under control conditions and after addition of 1 μM glacontryphan-M to the extracellular solution (Fig. 7, A and B). The peak value (I_p), the sustained component (I_sustained) and the charge (Q) of the Ca\textsuperscript{2+} current were measured. Application of glacontryphan-M dramatically reduced both peak and sustained Ca\textsuperscript{2+} current, an effect that partially could be recovered (Fig. 7A). On average glacontryphan-M produced a 30 ± 6% (p < 0.05; n = 5) reduction of I_p, a 43 ± 5% (p < 0.01; n = 5) decrease in I_sustained and a 24 ± 16% (p < 0.05; n = 5) reduction in the integrated Ca\textsuperscript{2+} current.

The observed reduction in the Ca\textsuperscript{2+} current was dose-dependent (Fig. 7C), and a maximum effect was observed at 1 μM glacontryphan-M. The concentration of glacontryphan-M at which half-maximal inhibition of the current was obtained was estimated using the Hill equation in Equation 1,

\[
\frac{Y}{Y_{\text{max}}} = \frac{C^n}{K_d + C^n}
\]

(Eq. 1)

where Y is the inhibition of peak Ca\textsuperscript{2+} current observed at the concentration C of glacontryphan-M, Y_{max} is the maximum inhibition of peak Ca\textsuperscript{2+} current, K_d is the association constant, and n is the cooperativity factor. The equation was fit to the data points yielding half-maximal inhibition of the Ca\textsuperscript{2+} current at 50 nM glacontryphan-M.

Contrary to the observed inhibition of the voltage-dependent Ca\textsuperscript{2+} current by glacontryphan-M, 1 μM glacontryphan-M, where the Gla residues have been replaced with Glu residues was without effect on the Ca\textsuperscript{2+} current (Fig. 7, D and E).

The Inhibitory Action of Glacontryphan-M on Ca\textsuperscript{2+} Current Is Dependent on the Presence of Extracellular Ca\textsuperscript{2+}—Above glacontryphan-M is described to possess calcium binding properties. To investigate if the apo form of glacontryphan-M could reduce the current through the Ca\textsuperscript{2+} channel, patch-clamp experiments were performed in the absence of Ca\textsuperscript{2+}. In a Ca\textsuperscript{2+}-free media the current through the channel is carried by other cations, mostly Na\textsuperscript{+}, which makes it possible to investigate the effect of inhibitors and potentiators on the channel also in the absence of Ca\textsuperscript{2+}. Inward cation currents were evoked by membrane depolarizations from −100 mV to −30 mV lasting 100 ms. Replacing the extracellular Ca\textsuperscript{2+} with sucrose to maintain osmolality, glacontryphan-M failed to exhibit inhibition of the voltage-dependent cation current (Fig. 8, A and B). On the contrary glacontryphan-M caused a small but significant increase in the inward peak-current of 22 ± 8% (p < 0.05, n = 7) compared with control. Glacontryphan-M was, as in the presence of Ca\textsuperscript{2+}, without effect on the cation currents evoked in the absence of Ca\textsuperscript{2+} (Fig. 8, C and D). Thus it can be suggested that the Ca\textsuperscript{2+}-bound form of glacontryphan-M acts as an antagonist on the Ca\textsuperscript{2+} channel activity whereas the Ca\textsuperscript{2+}-unbound form of glacontryphan-M has no or minor stimulatory effect on the channel activity. The structural investigation of glacontryphan-M described in the accompanying report (47) revealed calcium-induced conformational changes that might be necessary for the antagonistic effect.

Glacontryphan-M Blocks Currents through L-type Ca\textsuperscript{2+} Channels—Insulin secretion from mouse pancreatic B-cell depends to a large extent on the influx of Ca\textsuperscript{2+} through voltage-dependent Ca\textsuperscript{2+} channels, most likely L-type Ca\textsuperscript{2+} channels (39, 49). Currents through the L-type Ca\textsuperscript{2+} channels can be blocked by dihydropyrodines, phenylamines, and benzothiazepines. The only peptide toxin reported to specifically affect L-type calcium channels is the ω-conotoxin TxVII from C. textile (50). We therefore investigated if the Ca\textsuperscript{2+}-bound form of contrylan with a 51 residue signal/pro-peptide sequence and a 1 residue C-terminal extension (Gly; Fig. 6). In Table II the predicted translation product of glacontryphan-M is compared with the previously described contrylan sequences. The pro
eotelytic propeptide processing site usually consists of one or a pair of basic amino acids that immediately precede the mature toxin in the precursor sequence. In glacontryphan-M a standard signal for proteolysis is not found immediately before the mature peptide and a further proteolytic processing step at the C terminus may be required to generate the mature peptide. The C terminus of glacontryphan-M is presumably processed by conventional mechanisms to yield the amidated C-terminal cysteine residue.

The high density of arginine and lysine residues in the C-terminal portion of the propeptide region compared with the non-Gla-containing contrylans could indicate that these residues are part of the γ-carboxyl recognition site that distinguishes contrylan carboxylate substrates from contrylans that do not get carboxylated. Interestingly, this high frequency of basic residues also occurs in the propeptide sequences of the γ-carboxylated conopeptides e-TxIX (15) and tx9a (48) isolated from C. textile and conG (14) isolated from C. geographicus.

Effects of Glacontryphan-M on Inward Voltage-dependent Ca\textsuperscript{2+} Currents—The pharmacological properties of glacontryphan-M on voltage-dependent Ca\textsuperscript{2+} currents were investigated with the synthetic peptide. For this purpose patch-clamp measurements on single pancreatic B-cells using the perforated patch configuration were performed. To elicit Ca\textsuperscript{2+} currents 100-ma depolarizations from −70 to 0 mV were applied to the cell under control conditions and after addition of 1 μM glacontryphan-M to the extracellular solution (Fig. 7, A and B). The peak value (I_p), the sustained component (I_sustained) and the charge (Q) of the Ca\textsuperscript{2+} current were measured. Application of glacontryphan-M dramatically reduced both peak and sustained Ca\textsuperscript{2+} current, an effect that partially could be recovered (Fig. 7A). On average glacontryphan-M produced a 30 ± 6% (p < 0.05; n = 5) reduction of I_p, a 43 ± 5% (p < 0.01; n = 5) decrease in I_sustained and a 24 ± 16% (p < 0.05; n = 5) reduction in the integrated Ca\textsuperscript{2+} current.

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Contrary to the observed inhibition of the voltage-dependent Ca\textsuperscript{2+} current by glacontryphan-M, 1 μM glacontryphan-M, where the Gla residues have been replaced with Glu residues was without effect on the Ca\textsuperscript{2+} current (Fig. 7, D and E).

The Inhibitory Action of Glacontryphan-M on Ca\textsuperscript{2+} Current Is Dependent on the Presence of Extracellular Ca\textsuperscript{2+}—Above glacontryphan-M is described to possess calcium binding properties. To investigate if the apo form of glacontryphan-M could reduce the current through the Ca\textsuperscript{2+} channel, patch-clamp experiments were performed in the absence of Ca\textsuperscript{2+}. In a Ca\textsuperscript{2+}-free media the current through the channel is carried by other cations, mostly Na\textsuperscript{+}, which makes it possible to investigate the effect of inhibitors and potentiators on the channel also in the absence of Ca\textsuperscript{2+}. Inward cation currents were evoked by membrane depolarizations from −100 mV to −30 mV lasting 100 ms. Replacing the extracellular Ca\textsuperscript{2+} with sucrose to maintain osmolality, glacontryphan-M failed to exhibit inhibition of the voltage-dependent cation current (Fig. 8, A and B). On the contrary glacontryphan-M caused a small but significant increase in the inward peak-current of 22 ± 8% (p < 0.05, n = 7) compared with control. Glacontryphan-M was, as in the presence of Ca\textsuperscript{2+}, without effect on the cation currents evoked in the absence of Ca\textsuperscript{2+} (Fig. 8, C and D). Thus it can be suggested that the Ca\textsuperscript{2+}-bound form of glacontryphan-M acts as an antagonist on the Ca\textsuperscript{2+} channel activity whereas the Ca\textsuperscript{2+}-unbound form of glacontryphan-M has no or minor stimulatory effect on the channel activity. The structural investigation of glacontryphan-M described in the accompanying report (47) revealed calcium-induced conformational changes that might be necessary for the antagonistic effect.

Glacontryphan-M Blocks Currents through L-type Ca\textsuperscript{2+} Channels—Insulin secretion from mouse pancreatic B-cell depends to a large extent on the influx of Ca\textsuperscript{2+} through voltage-dependent Ca\textsuperscript{2+} channels, most likely L-type Ca\textsuperscript{2+} channels (39, 49). Currents through the L-type Ca\textsuperscript{2+} channels can be blocked by dihydropyrodines, phenylamines, and benzothiazepines. The only peptide toxin reported to specifically affect L-type calcium channels is the ω-conotoxin TxVII from C. textile (50). We therefore investigated if the Ca\textsuperscript{2+}-bound form of
glacontryphan-M could be a possible candidate as a new antagonist for the L-type Ca\textsuperscript{2+} channels.

In Fig. 9A voltage clamp depolarizations were applied to a single B-cell to evoke inward Ca\textsuperscript{2+} currents under control conditions, after addition of 5 \mu M of the dihydropyridine isradipine in the extracellular solution and after application of 1 \mu M glacontryphan-M in the continued presence of isradipine. Isradipine reduced the peak Ca\textsuperscript{2+} current by 30\% (p < 0.01, n = 10; Fig. 9B), which is similar to what has been published before in pancreatic B-cells (49). Glacontryphan-M failed to inhibit the current further in the presence of isradipine, suggesting that glacontryphan-M is an L-type channel antagonist.

DISCUSSION

This report describes the purification, characterization, cloning, and mode of action of a novel C. marmoreus conotoxin (glacontryphan-M) that is a member of the contryphan family. Glacontryphan-M is the first contryphan reported to act as a specific L-type calcium channel blocker. As with most other contryphans, glacontryphan-M contains a post-translationally

**Fig. 5.** Ca\textsuperscript{2+} binding studies of glacontryphan-M. A and B, calcium-induced changes in the intrinsic emission spectra of glacontryphan-M (GlaconTrp-M) and glucontryphan-M (GluconTrp-M), respectively. Corrected emission spectra were obtained at 20 °C in 50 mM Tris-HCl, 0.1 mM NaCl, pH 7.5 in the absence (---) or presence (- - -) of CaCl\textsubscript{2} (10.2 mM in A and 22.0 mM in B). C and D, changes in the intrinsic fluorescence of glacontryphan-M and glucontryphan-M, respectively, as a function of the CaCl\textsubscript{2} concentration. F\textsubscript{0} and F are the emission intensities in the absence and in the presence of calcium, respectively. The peptide concentrations were 2 \mu M.

**Fig. 6.** Nucleotide sequence of cDNA encoding the precursor form of glacontryphan-M and its predicted translation product, prepro-glacontryphan-M. The mature conopeptide is shown in *bold type*. The positions of the oligonucleotides used are marked with arrows.
isomerized D-Trp, is amidated at the C terminus and follows the conserved contrphan sequence motif CPxXPXC (where x and X represents any D- and L- handed residues, respectively). On the other hand, glacontryphan-M diverges from known contrphans by the presence of two Gla residues (Gla2 and Gla4), by having an extended N terminus, and by the occurrence of an intercysteine loop histidine residue (His8).

The post-translational formation of D-amino acids increases the diversity of products that can be synthesized from one gene. The mechanism of isomerization of L- to D- amino acids in invertebrate peptides has been suggested to be a position-dependent process that occurs at a late stage during synthesis, after cleavage of peptide precursor sequences (51). A position-specific rule could however not apply for the contrphans since in glacontryphan-M, the D-tryptophan occurs at position seven whereas in the other reported contrphans the D-amino acid occurs at position three, four, or five. The presence of D-amino acids in certain peptides has been reported to be essential for their biological activity, but also to increase their stability since peptide bonds with the D-amino acid are more resistant to proteases (51). Efforts to gain insight into the importance of the D-Trp in glacontryphan-M failed due to the exclusive formation of peptide dimers in the folding process of the synthetic L-Trp isomer.

Glacontryphan-M is the first contrphan peptide reported to carry the post-translationally modified amino acid Gla, providing it with calcium binding properties. The sole example of

### Table II

| Peptide          | Sequencea |
|------------------|-----------|
| Contryphan-R/Tx  | MGKTLTILVLAVALLCCSTQMAAQGDGDQPAARNAVPRODNPDPGSAKFMNVQRRGCPWEPWCG |
| Contryphan-Tx    | MGKTLTILVLAVALLCCSTQMAAQGDGDQPAARNAVPRODNPDPGSAKFMNVQRRGCPWEPWCG |
| Leu-contrphan-Tx | MGKTLTILVLAVALLCCSTQMAAQGDGDQPAARNAVPRODNPDPGSAKFMNVQRRGCPWEPWCG |
| Contryphan-R     | MGKTLTILVLAVALLCCSTQMAAQGDGDQPAARNAVPRODNPDPGSAKFMNVQRRGCPWEPWCG |
| Contryphan-Sm    | MGKTLTILVLAVALLCCSTQMAAQGDGDQPAARNAVPRODNPDPGSAKFMNVQRRGCPWEPWCG |
| Contryphan-P     | MGKTLTILVLAVALLCCSTQMAAQGDGDQPAARNAVPRODNPDPGSAKFMNVQRRGCPWEPWCG |
| Glacontryphan-M  | MGKTLTILVLAVALLCCSTQMAAQGDGDQPAARNAVPRODNPDPGSAKFMNVQRRGCPWEPWCG |

*a* Bold regions correspond to mature peptide sequences. Stars indicate unique amino acid residues in Glacontryphan-M.
γ-carboxyglutamic acid in invertebrates is in the Conus peptides. The function of Gla in the conotoxins is uncertain, although the presence of γ-carboxyglutamic acid has been shown to be a requirement for the biological activity in the Conus-derived Gla-rich conantokin G (5, 29, 30). Miles et al. (52), however, have suggested that the two Gla residues in conotoxin tx9a, from C. textile, are not crucial for the biological activity of this peptide. In the present study, electrophysiological experiments carried out on mouse pancreatic B-cells showed that glacontryphan-M specifically blocks L-type calcium channel activity. Substitutions of the Gla residues with Glu residues resulted in a peptide (glucontryphan-M) lacking the calcium ion channel blocking effect. This implies an essential role for the Gla residues in glacontryphan-M for biological activity.

Using fluorescence spectroscopy glacontryphan-M was demonstrated to bind calcium with a \( K_D \) of 0.63 mM. In the conotoxins the metal binding properties (22, 27, 28) and the three-dimensional structures have suggested a specific structural role for Gla in the conopeptides (20, 23–26). Conantokins G and T were demonstrated to be susceptible to conformational change as a result of calcium binding to Gla residues (22). The calcium-dependent intrinsic fluorescence quench was clearly impaired in glacontryphan-M implying the Gla residues in glacontryphan-M to be involved in the calcium interaction. In order to further elucidate the role of Gla in glacontryphan-M and the role of calcium binding to the peptide, the high resolution three-dimensional structure of the peptide in the presence and absence of metal ions has been determined (47).

Regulated secretion in neurons and neuroendocrine cells depends to a large extent on influx through voltage-dependent Ca\(^{2+}\) channels (38). This is also the situation in the mouse pancreatic B-cells releasing insulin (39, 49). Approximately 50% of the Ca\(^{2+}\) current in the pancreatic B-cells is generated by influx of Ca\(^{2+}\) through L-type Ca\(^{2+}\) channels (Ca \(V_1.2\)) (49). The L-type Ca\(^{2+}\) channels and the secretory granules co-localize in the B-cell (45, 53), and it has been demonstrated that the L-type Ca\(^{2+}\) channels bind to SNARE proteins (54), which makes the L-type Ca\(^{2+}\) channel extremely important for rapid insulin secretion. Currents through the L-type Ca\(^{2+}\) channels can be blocked by dihydropyridines, phenylamines, and benzothiazepines whereas the N-type (Ca \(V_2.2\), P/Q-type (Ca \(V_2.1\)) Ca\(^{2+}\) channels, which are found primarily in neurons, are blocked by polypeptides of different toxins from snail and spider venoms (for review see Ref. 55). Except for \(ω\)-conotoxin VII derived from C. textile, glacontryphan-M is the only known peptide toxin that specifically blocks L-type Ca\(^{2+}\) channels.
The antagonistic action of glyctryphan-M on the Ca\(^{2+}\) currents of B-cells was shown to be dependent on the presence of extracellular Ca\(^{2+}\). It is thus possible that binding of Ca\(^{2+}\) to the Gla residues in glyctryphan-M contributes to its ability to adopt a defined conformation, and this divergent cation-dependent conformation is necessary for the interaction with the Ca\(^{2+}\) ion channels. The calcium-induced structural perturbations of glyctryphan-M that are functionally necessary have been further investigated and are discussed in Grant et al. (47).

In a very recent report K\(^{+}\) channels were demonstrated as the molecular target of Contryphan-V\(n\) (37). No effect was however observed on calcium ion currents. A sequence comparison of glyctryphan-M with Contryphan-V\(n\) shows that the two peptides differ at their N termini and at one amino acid position in their intercysteine loops suggesting these residues to be important for ion channel binding. It would be interesting to further investigate the molecular recognition properties of the known Contryphan peptides.

cDNA cloning of glyctryphan-M deduced a signal/propeptide sequence that is homologous to earlier reported sequences of Contryphan except for the 10 amino acids preceding the mature toxin region. The non-homologous part of the glyctryphan-M peptide could thus be important for substrate recognition by the Conus carboxylase. The \(\gamma\)-glutamyl recognition sequence was originally defined for the mammalian enzyme (3, 58). Cloning of the cDNA encoding the precursor form of Conantokin-G to the Gla-containing Conus propeptide sequence of conantokin-G to the Gla-containing Glacontryphan-M with Contryphan-V\(n\) shows that the two peptides differ in their C-terminal and at one amino acid position in their intercysteine loops suggesting these residues to be important for ion channel binding. It would be interesting to further investigate the molecular recognition properties of the known Contryphan peptides.

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32463

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The First γ-Carboxyglutamic Acid-containing Contryphan: A SELECTIVE L-TYPE CALCIUM ION CHANNEL BLOCKER ISOLATED FROM THE VENOM OF CONUS MARMOREUS

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