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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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Contryphans 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 tryptophan 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 carboxylated 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-D-Trp-His-Pro-Trp-Cys. As with most other contryphans, glacontryphan-M is amidated at the C terminus and maintains the five-residue intercysteine loop. The occurrence of a D-tryptophan 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 mM. Cloning of the full-length cDNA encoding glacontryphan-M revealed that the primary translation product carries an 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)[1] 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 piscivo-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY485226.

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[1] The abbreviations used are: Gla, γ-carboxyglutamic acid; HPLC, high performance liquid chromatography; NanoESI-MS, nanoelectrospray ionization mass spectrometry.

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rous (31, 32), molluscivorous (33), and vermivourous (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. Contryphans 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 contryphan was reported (37) as Contryphan-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 contryphan peptide, glacontryphan-M, extracted from the venom of the molluscivorous *C. marmoreus*. Glacontryphan-M is the first example of a contryphan 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 propeptide regions of the earlier described contryphans. 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) exocytosis from pancreatic B-cells is determined by calcium influx through voltage-dependent Ca^{2+} channels (39). This is the first Gla-containing conopeptide reported to modulate the activity of this type of calcium ion 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 × 92 cm) equilibrated with 0.2 M ammonium acetate buffer (pH 7.5) and eluted with a flow rate of 10.3 ml/h (Fig. 1*A*). The material in the major Gla-containing peak was separated on a reversed-phase HPLC column (HyChrom C18, 5 μm; 10 × 250 mm) in 0.1% (v/v) trifluoroacetic acid and eluted with a linear acetonitrile gradient at a flow rate of 2 ml/min (Fig. 1*B*). A total of 30 nmol of Glacontryphan-M was obtained from a second reversed-phase HPLC purification step (Vydac C18 column, 5 μm; 4.6 × 250 mm) in 0.1% trifluoroacetic acid using a linear acetonitrile gradient at a flow rate of 0.5 ml/min (Fig. 1*C*).

Amino Acid Analysis and Sequencing—The amino acid composition of glacontryphan-M was determined after acid hydrolysis, except for Gla, which was measured after alkaline hydrolysis (40, 41). For peptide sequencing a PerkinElmer Life Sciences (Foster City, CA) ABI Procise 494 sequencer was used according to the protocol from the manufacturer. To identify the Gla residues in the sequence, the sample was methyl-esterfied with methanolic HCl prior to sequencing (42).

Reduction and Alkylation of Disulfide Bonds—For reduction and alkylation of cysteine residues with iodoacetic acid (BDH Chemicals Ltd, Poole, England), the peptides were dissolved in 250 μl of 6 M guanidine hydrochloride in 1 M Tris-HCl, 10 mM EDTA (pH 8.6). Dithiothreitol (ICN Biomedicals Inc.) was added to 20 mM followed by incubation at 37 °C for 2 h, after which iodoacetic acid was added to a final concentration of 50 mM. After incubation at room temperature for 30 min, β-mercaptoethanol (BDH Chemicals Ltd) 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.

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 DPfd L- 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% anhydrous trifluoroacetic acid containing relevant scavengers. Each peptide was then applied to a C_8 preparative reversed-phase HPLC 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 mM iodine in 20% acetonitrile in 0.1% (v/v) trifluoroacetic acid. After 15 min the reactions were quenched with a few drops of 0.1 M ascorbic acid. Each of the oxidized peptides was applied to a reversed-phase C_8 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 D-tryptophan-containing peptide was further purified on a reversed-phase C_{18} 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 L-tryptophan-containing peptide, NanoESI-MS analysis revealed that only dimers had formed during the folding step. A peptide, glucontryphan-M, covering the mature toxin region of glacontryphan-M but having the Gla residues at positions 2 and 4 replaced with glutamic acid 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 quadrapole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, 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 liter/min. The acquisition and the deconvolution of data were performed on an AnalystQS Windows PC data system. Bioanalyst version 1.0 software (Applied Biosystems/MDS Sciex) was used to analyze the mass spectra. Samples were dried and dissolved (~1–5 pmol/μl) in 50% (v/v) methanol supplied with 1% (v/v) formic acid for NanoESI-MS and MS/MS analysis in positive ion mode and in 50% acetonitrile for NanoESI-MS analysis in negative ion mode. Parent ions (identified in a time-of-flight MS survey scan) for MS/MS analysis were selected in Q1, and product ions were generated in Q2 using N_2 as the collision gas and collision energies of 14–35 eV.

Identification and Sequencing of a cDNA Clone Encoding Glacontryphan-M—On the basis of the amino acid sequence of the isolated contryphan peptide from *C. marmoreus* and the well conserved signal peptide of the previously identified contryphans, oligonucleotide primers were designed for PCR (amplification of the corresponding cDNA from a Lambda ZAP II Custom cDNA library (Stratagene). Two oligonucleotide primers with degenerate nucleotide sequences were synthesized. p95–1, 5′-ATG GGN AAR YTN ACN ATH YTN G-3′; p95–2, 5′-CCR CAC CAN GGR TGC CAN GGR CAY TC-3′(where *n* = A, T, C or G, Y = T or C, H = A, C or T, *r* = A or G) represent sequences complementary to the sequences at the N terminus signal peptide and the C terminus toxin region of the peptide, respectively. The amplification parameters for the PCR reactions were 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 46 °C for 1 min and 72 °C for 1 min; and a 10-min extension at 72 °C. The PCR product was TA-cloned into the pGEM-T vector (Promega), and the resulting plasmid DNA sequenced using M13 forward, 5′-GTT TTC CCA GTC ACG AC-3′ and reversed 5′-CAG GAA ACA GCT ATG AC-3′, primers. Gene-specific oligonucleotide primers, p95–3, 5′-TCT CGC TCT ACC TGG GTT ATC GTC-3′and p95–4, 5′-AGC GAG AAG AAA GCG CAT GAA AG-3′ (Fig. 6) were used in combination with the vector-specific M13 forward and reverse primers for PCR amplification of the 5′- and the 3′-ends of the cDNA encoding glacontryphan-M, respectively. The PCR products were cloned in vector pGEM-T and sequenced using M13 reverse and forward primers. The entire cDNA sequence of glacontryphan-M was assembled from the overlapping sequences and is predicted to contain the amino acid sequence of the mature contryphan toxin. The obtained cDNA sequence was confirmed by cloning (in pGEM-T) and sequencing (using M13 reverse and forward primers) of the PCR products obtained with oligonucleotide primer p95–6, 5′-AAC CTT TAT CAT GG-3′, in combination with primer p95–7, 5′-CAA TCG TGG ATT CCG ATC-3′, or primer p95–8, 5′-GGA CAT TCA GAC TCA TTT AG-3′ (Fig. 6).

Calcium Binding Measurements—Tryptophan fluorescence was measured at 20 °C in a Spex Fluoromax-3 spectrofluorometer (Jobin Yvon-Spex, Instruments s.a., Inc.) equipped with a 150 watt Xenon lamp. The peptide concentrations were 2 μM, 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 photodecomposition. Fluorescence emission spectra were recorded between 295 and 425 nm with an excitation wavelength of 280 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 μl portions of 0.11 or 0.77 mM stock solutions of $CaCl_2$ to 1 ml of sample

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and by averaging 30 signals readings of 0.25 s each. The excitation wavelength was 280 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 $1 - F/F_0$ where F and F_0 are the fluorescence intensities in the presence and absence of Ca^{2+} 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 Ca^{2+}-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 sylgard 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 $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES (pH 7.4 using NaOH). In the Ca^{2+}-free extracellular solution $CaCl_2$ was substituted with 11.4 mM sucrose (to retain similar osmolarity) and 2 mM EGTA (to bind Ca^{2+}). Glacontryphan-M, glucontryphan-M, and isradipine were added to the extracellular solution as indicated in the figures. The pipette solution contained 76 mM Cs_2SO_4, 10 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES (pH 7.35 using CsOH). Electrical contact was established by addition of the pore-forming compound amphotericin 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. marmoreus* venom was extracted and chromatographed on a Sephadex G-50 superfine column (Fig. 1*A*). 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-carboxyglutamic acid residues were identified in a peptide that had been methylesterfied 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-Pro-Trp-His-Pro-Trp-Cys. The sequence shows high similarity to the amino acid sequences earlier described for peptides of the contryphan family of conotoxins (Table I). To further investigate the primary sequence of glacontryphan-M the peptide was analyzed by NanoESI-MS.

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. 2*A* (*m/z* 1400–1480) and *B* (*m/z* 680–750). The signal observed at m/z 1470.47 Da (M-1H, Fig. 2*A*) corresponds to the molecular ion of the peptide and the major ion peak at m/z 734.74 Da (Fig. 2*B*), 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. 2*B*). This loss of CO_2 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. 2*C*). 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. 3*A*). 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

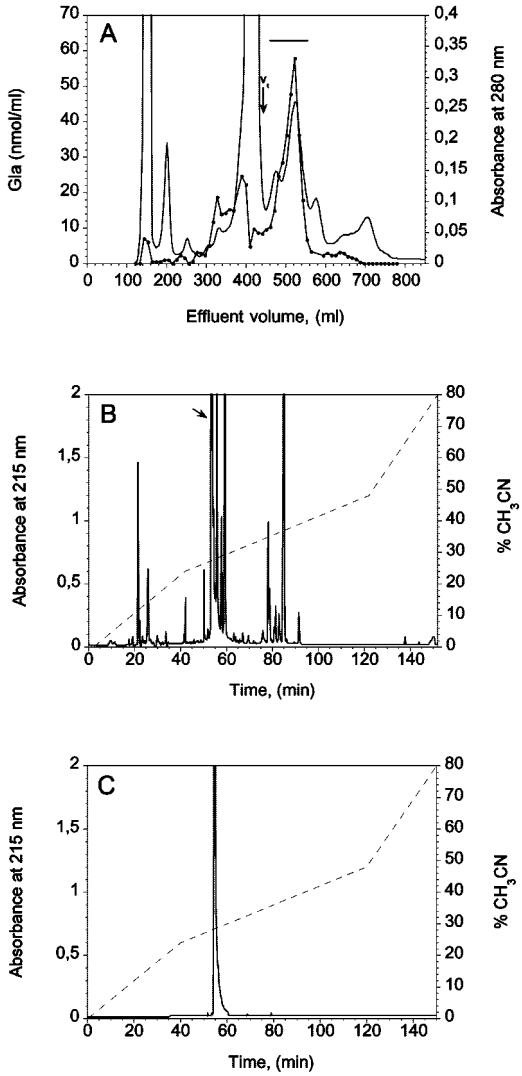

FIG. 1. **Purification of glacontryphan-M from the venom of *C. marmoreus*.** *A*, *Conus* venom was extracted and chromatographed on a column of Sephadex G50 Superfine. (—) denotes A_{280}. The major Gla-containing peak that contained peptide glacontryphan-M is denoted by a *horizontal bar*. The *arrow* marked V_t denotes one column volume. Every third collected fraction was analyzed for its content of γ-carboxyglutamic acid (—●—). *B* and *C*, isolation of glacontryphan-M (peak denoted by *arrow*) by HPLC separation on two subsequent C_{18} columns in 0.1% trifluoroacetic acid and eluted with acetonitrile gradients (—).

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TABLE I
Comparison of mature contryphan sequences

Peptide	Sequence[a]	Species	Ref.
Des(Gly[1])contryphan-R	COWEPWC-NH_2	*C. radiatus*	35
Contryphan-R	GCOWEPWC-NH_2	*C. radiatus*	35
Bromocontyphan-R	GCOWEPXC-NH_2	*C. radiatus*	31
Contryphan-Sm	GCOWQPWC-NH_2	*C. stercusmuscarum*	32
Contryphan-P	GCOWDPWC-NH_2	*C. purpurascens*	32
Contryphan-R/Tx	GCOWEPWC-NH_2	*C. textile*	33
Contryphan-Tx	GCOWQPYC-NH_2	*C. textile*	33
Contryphan-Vn	GDCPWKPWC-NH_2	*C. ventricosus*	34
Leu-contryphan-P	GCVLLPWC-OH	*C. purpurascens*	36
Leu-contryphan-Tx	CVLYPWC-NH_2	*C. textile*	33
Glacontryphan-M	NγSγCPWHPWC-NH_2	*C. marmoreus*	This work

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

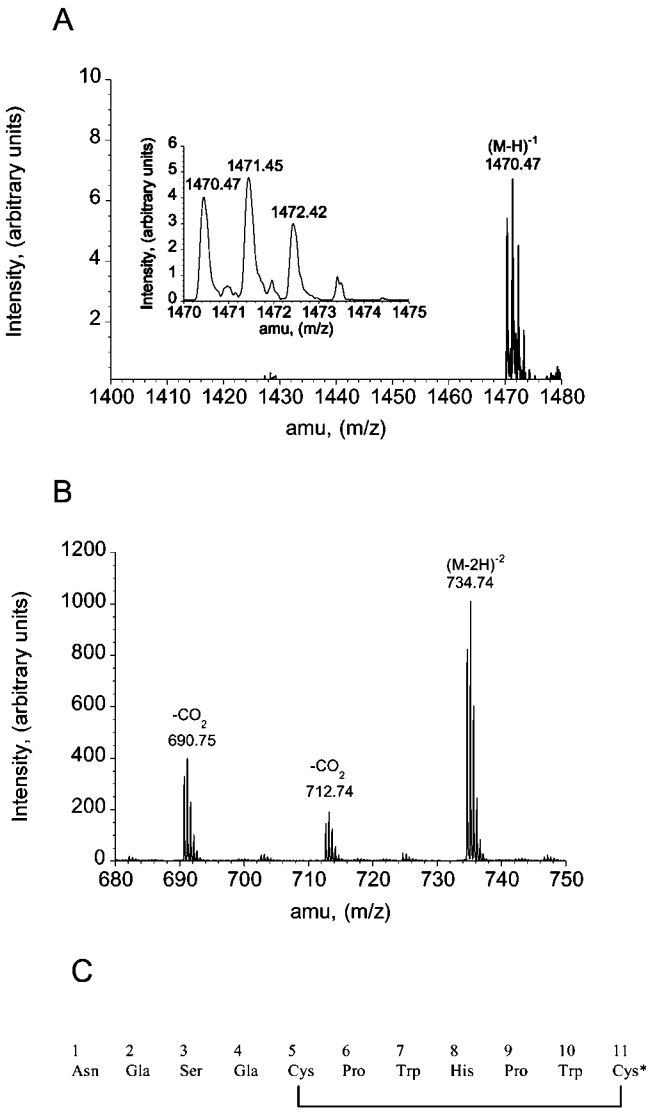

FIG. 2. **Negative-ion ESI mass spectra of native glacontryphan-M.** *A* and *B*, monoisotopic molecular mass of glacontryphan-M was determined from the ESI-MS spectra to be 1471.47 Da. These data fit with the calculated mass (1471.48 Da) of glacontryphan-M having an amide group at its C terminus and having the cysteines disulfide-bonded. The presence of Gla in the sequence is notable in the mass spectra via decarboxylation of the peptide resulting in a loss of 44 Da for each Gla residue. The sequence of glacontryphan-M is shown in C. * denotes that the C-terminal carboxyl group of the peptide is amidated.

795.80 Da (Fig. 3*B*) 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. 3*B*). 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 isoptope 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. 4*C*). 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 Ca^{2+} binding of glacontryphan-M. Calcium binding was found to generate a 76% reduction of the intrinsic tryptophan fluorescence (Fig. 5*A*). The addition of Ca^{2+} 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 Ca^{2+} binding (Fig. 5*B*). The changes in fluorescence emission as a function of Ca^{2+} concentration for glacontryphan-M were adequately described by a one-site binding equation giving an apparent K_D of 0.63 mM (Fig. 5*C*). 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* cDNA library. The cDNA sequence predicts a primary translation product of 63 amino acids that corresponds to the 11 residues of

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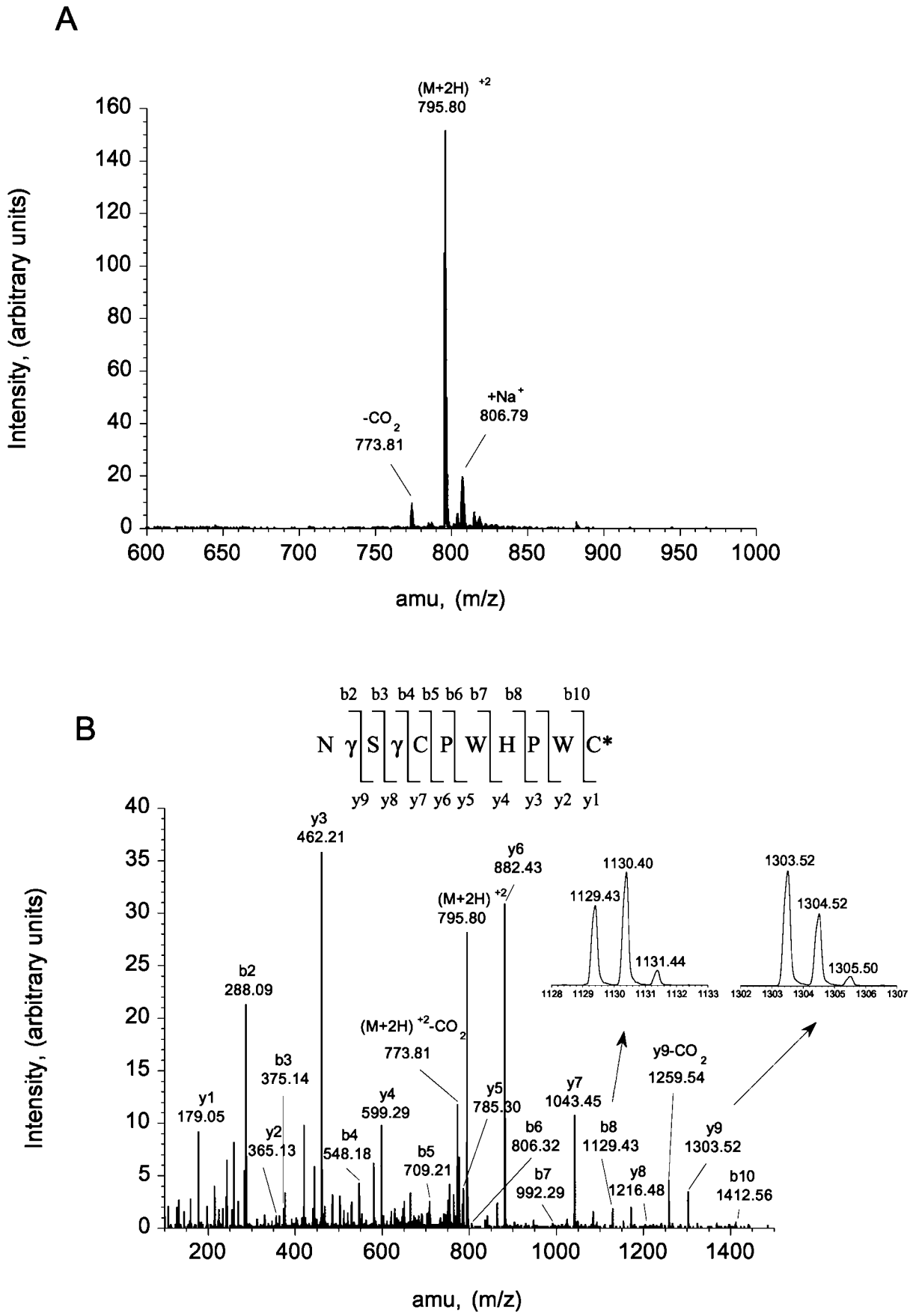

FIG. 3. **Positive-ion ESI-MS and MS/MS spectra of *S*-carboxymethylated glacontryphan-M.** *A*, monoisotopic molecular mass of the *S*-carboxymethylated glacontryphan-M was determined from the mass spectrum to be 1589.60. *B*, ESI MS/MS product ion spectrum of the doubly charged ion at *m/z* 795.80. 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.

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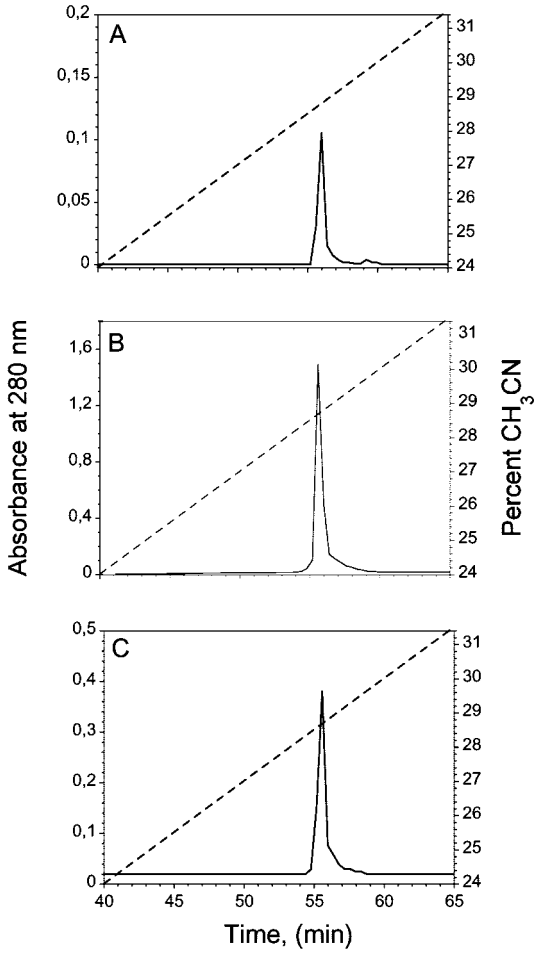

FIG. 4. **HPLC elution profiles.** Elution of (*A*), natural glacontryphan-M and (*B*), synthetic glacontryphan-M containing a D-tryptophan in position 7 and an amidated C terminus. *C*, synthetic D-tryptophan containing peptide coelutes with the natural peptide isolated from *C. marmoreus*. HPLC runs were performed as described under "Experimental Procedures."

contryphan 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 contryphan sequences. The proteolytic 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 contryphans could indicate that these residues are part of the γ-carboxyl recognition site that distinguishes contryphan carboxylate substrates from contryphans that do not get carboxylated. Interestingly, this high frequency of basic residues also occurs in the propeptide sequences of the γ-carboxylated conopeptides ϵ-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^{2+} Currents—The pharmacological properties of glacontryphan-M on voltage-dependent Ca^{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^{2+} currents 100-ms 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_{sus}) and the charge (Q) of the Ca^{2+} current were measured. Application of glacontryphan-M dramatically reduced both peak and sustained Ca^{2+} current, an effect that partially could be recovered (Fig. 7*A*). 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_{sus} and a 24 ± 16% ($p < 0.05$; $n = 5$) reduction in the integrated Ca^{2+} current.

The observed reduction in the Ca^{2+} current was dose-dependent (Fig. 7*C*), 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,

$$\frac{Y}{Y_{max}} = \frac{C^n}{K_d + C^n} \qquad \text{(Eq. 1)}$$

where Y is the inhibition of peak Ca^{2+} current observed at the concentration C of glacontryphan-M, Y_{max} is the maximum inhibition of peak Ca^{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^{2+} current at 50 nM glacontryphan-M.

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

The Inhibitory Action of Glacontryphan-M on Ca^{2+} Current Is Dependent on the Presence of Extracellular Ca^{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^{2+} channel, patch-clamp experiments were performed in the absence of Ca^{2+}. In a Ca^{2+}-free media the current through the channel is carried by other cations, mostly Na^+, which makes it possible to investigate the effect of inhibitors and potentiators on the channel also in the absence of Ca^{2+}. Inward cation currents were evoked by membrane depolarizations from −100 mV to −30 mV lasting 100 ms. Replacing the extracellular Ca^{2+} with sucrose to maintain osmolarity, 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. Glucontryphan-M was, as in the presence of Ca^{2+}, without effect on the cation currents evoked in the absence of Ca^{2+} (Fig. 8, *C* and *D*). Thus it can be suggested that the Ca^{2+}-bound form of glacontryphan-M acts as an antagonist on the Ca^{2+} channel activity whereas the Ca^{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^{2+} Channels—Insulin secretion from mouse pancreatic B-cell depends to a large extent on the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, most likely L-type Ca_v 2.1 Ca^{2+} channels (39, 49). Currents through the L-type Ca^{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^{2+}-bound form of

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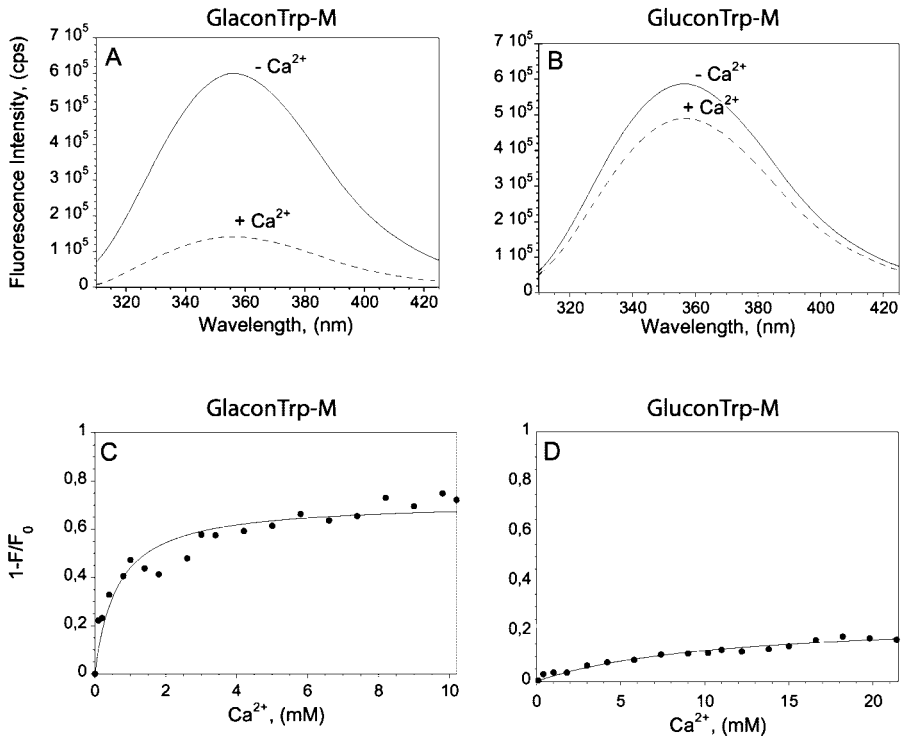

FIG. 5. **Ca^{2+} binding studies of glacontryphan-M.** *A* and *B*, calcium induced changes in the intrinsic emission spectra of glacontryphan-M (*Glacon-Trp-M*) and gluconcryphan-M (*Glucon-Trp-M*), respectively. Corrected emission spectra were obtained at 20 °C in 50 mM Tris-HCl, 0.1 M NaCl, pH 7.5 in the absence (—) or presence (- - -) of $CaCl_2$ (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 $CaCl_2$ concentration. F_0 and F are the emission intensities in the absence and in the presence of calcium, respectively. The peptide concentrations were 2 μM.

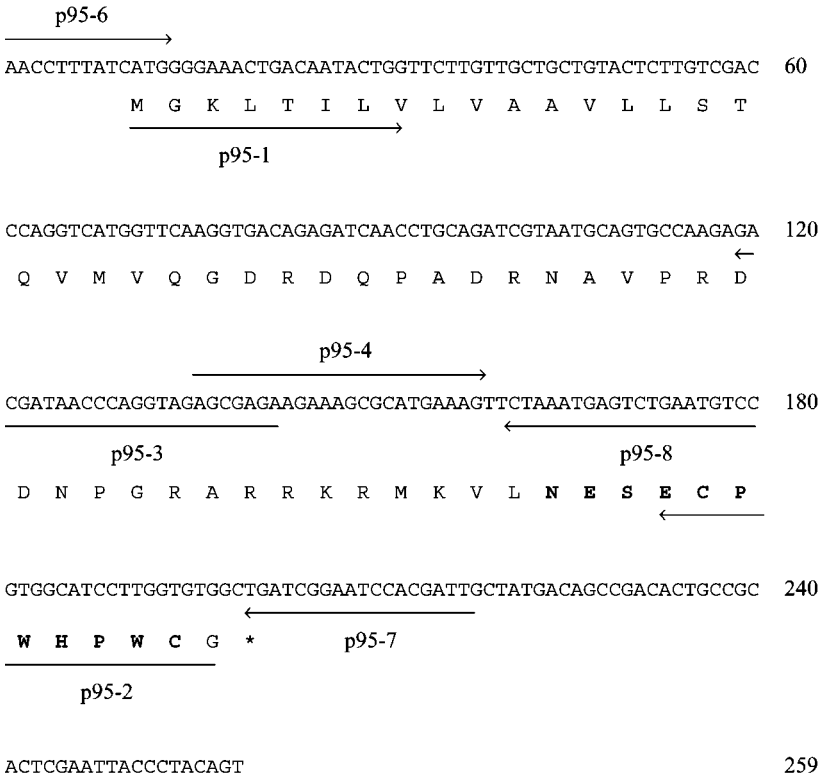

FIG. 6. **Nucleotide sequence of cDNA encoding the precursor form of glacontryphan-M and its predicted translation product, prepro-glacontryphan-M.** The mature conopeptide is shown in *bold type*. The positions of the oligonucleotides used are marked with *arrows*.

glacontryphan-M could be a possible candidate as a new antagonist for the L-type Ca^{2+} channels.

In Fig. 9*A* voltage clamp depolarizations were applied to a single B-cell to evoke inward Ca^{2+} currents under control conditions, after addition of 5 μM of the dihydropyridine isradipine in the extracellular solution and after application of 1 μM glacontryphan-M in the continued presence of isradipine. Isradipine reduced the peak Ca^{2+} current by 30 ± 5% ($p < 0.01$, $n = 10$; Fig. 9*B*), 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

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TABLE II
Contryphan prepropeptide sequences

Peptide	Sequence[a]
Contryphan-R/Tx	MGKLTILVLVAAVLLSTQAMAQGDGDQPAARNAVPRDDNPDGPSAKFMNVQRRS**GCPWEPWC**G
Contryphan-Tx	MGKLTILVLVAAVLLSTQVMVQGDGDQPADRDAVPRDDNPGGMSEKFLNALQRR**GCPWQPYC**G
Leu-contryphan-Tx	MGKLTILVLVAAVLLSAQVMVQGDGDQPADRKAVPREDNPGGASGKLMDVLRPKK**CVLYPWC**G
Contryphan-R	MGKLTILVLVAAVLLSAQVMVQGDGDQPADRNAVPRDDNPGGASGKFMNVLRRS**GCPWEPWC**G
Contryphan-Sm	MGKLTILVLVAAVLLSTQVMVQGDADQPADRDAVPRDDNPAGTDGKPMNVLRRF**GCPWQPWC**G
Contryphan-P	MGKLTILVLVAAVLLSTQVMVQGDGDQPAYRNAVPRDDNPGGAIGKFMNVLRRS**GCPWDPWC**G
Glacontryphan-M	MGKLTILVLVAAVLLSTQVMVQGDRDQPADRNAVPRDDNPGRARRKRMKVL**NESECPWHPWC**G
	* * ** * * ** *

[a] Bold regions correspond to mature peptide sequences. Stars indicate unique amino acid residues in Glacontryphan-M.

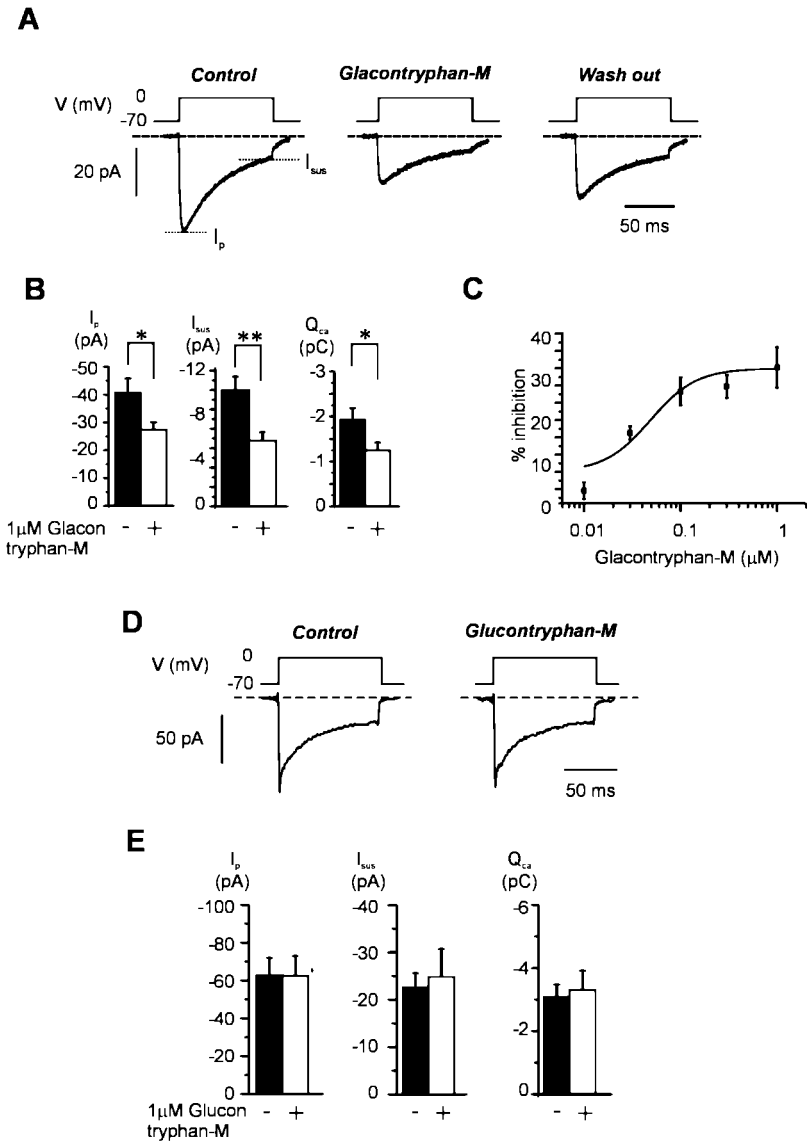

FIG. 7. **Inhibitory effects of glacontryphan-M on B-cell Ca^{2+} currents.** *A*, whole-cell Ca^{2+} currents from a single B-cell evoked by a 100 ms depolarization from −70 to 0 mV under control conditions (which contains 2.6 μM $CaCl_2$), 2 min after addition of 1 μM glacontryphan-M and 12 min after return to the control solution. *B*, average peak Ca^{2+} current (I_p), sustained Ca^{2+} current (I_{sus}), and integrated Ca^{2+} current (Q_{ca}) in the absence (*black bar*) or presence (*white bar*) of glacontryphan-M. I_p and I_{sus} were measured as indicated in *A*. Data are mean values ± S.E. of five paired experiments. *, $p < 0.05$ and **, $p < 0.01$. *C*, concentration dependence of the inhibitory action of glacontryphan-M on peak Ca^{2+} currents. The curve represents a fit of Equation 1 to the data points. Data are mean ± S.E. of 5–7 experiments. *D*, Ca^{2+} currents evoked by a 100-ms depolarization under control conditions and 2 min after addition of 1 μM glucontryphan-M. Notice the lack of inhibition when substituting glacontryphan-M with glucontryphan-M. *E*, mean data of the experiments in *D*. The values of peak Ca^{2+} current (I_p), sustained Ca^{2+} current (I_{sus}), and integrated Ca^{2+} current (Q_{ca}) are mean ± S.E. in seven paired experiments.

isomerized D-Trp, is amidated at the C terminus and follows the conserved contryphan sequence motif CP*x*XP*X*C (where *x* and *X* represents any D- and L- handed residues, respectively). On the other hand, glacontryphan-M diverges from known contryphans by the presence of two Gla residues (Gla^2 and Gla^4), by having an extended N terminus, and by the occurrence of an intercysteine loop histidine residue (His^8).

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 contryphans since in glacontryphan-M, the D-tryptophan occurs at position seven whereas in the other reported contryphans 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 contryphan peptide reported to carry the post-translationally modified amino acid Gla, providing it with calcium binding properties. The sole example of

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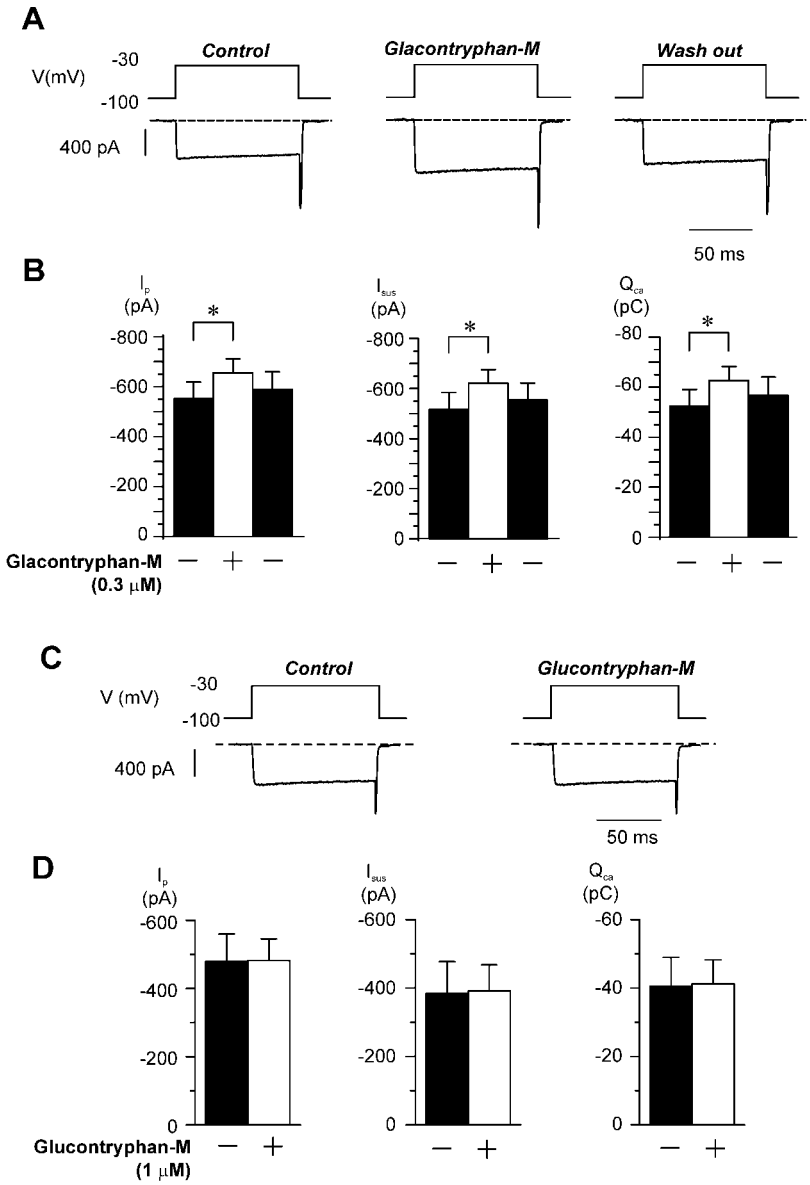

FIG. 8. **The inhibitory action of glacontryphan-M on Ca^{2+} current needs the presence of extracellular Ca^{2+}.** *A*, whole cell inward cation currents evoked by a 100-ms depolarization from −100 to −30 mV in a Ca^{2+} free extracellular solution under control conditions, 2 min after application of 0.3 μM glacontryphan-M and 12 min after washout. *B*, mean amplitude of peak Ca^{2+} current (I_p), sustained Ca^{2+} current (I_{sus}), and the integrated Ca^{2+} currents in the absence of Ca^{2+} before (*black bar*), 2 min after addition of glacontryphan-M (*white bar*) and 12 min after withdrawal of glacontryphan-M (*gray bar*). Data are mean values ± S.E. of seven paired experiments. *, $p < 0.05$. *C*, lack of effect of 1 μM glacontryphan-M on inward cation currents. Experiments were conducted under the same conditions as in *A* but glacontryphan was substituted for glucontryphan-M. *D*, mean data of the experiments in *C*. Data are mean values ± S.E. of seven paired experiments.

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 conantokins 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 glucontryphan-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 dihydropyrodines, 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.

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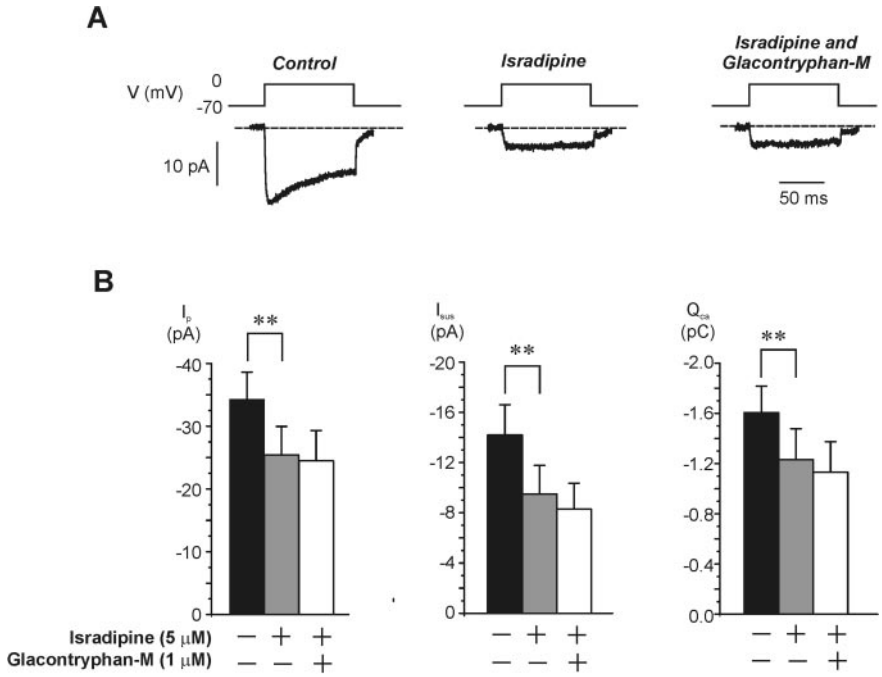

FIG. 9. **Glacontryphan-M blocks L-type Ca^{2+} channels.** *A*, whole cell Ca^{2+} currents recorded under control conditions, 2 min after application of 5 μM of the L-type channel antagonist isradipine and 2 min after addition of 1 μM glacontryphan-M in the continued presence of isradipine. *B*, mean data of experiments in *A*. Data are mean values ± S.E. of 10 paired experiments. **, $p < 0.01$.

The antagonistic action of glacontryphan-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 glacontryphan-M contributes to its ability to adopt a defined conformation, and this divalent cation-dependent conformation is necessary for the interaction with the Ca^{2+} ion channels. The calcium-induced structural perturbations of glacontryphan-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-Vn (37). No effect was however observed on calcium ion currents. A sequence comparison of glacontryphan-M with contryphan-Vn 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 glacontryphan-M deduced a signal/propeptide sequence that is homologous to earlier reported sequences of contryphans except for the 10 amino acids preceding the mature toxin region. The non-homologous part of the glacontryphan-M peptide could thus be important for substrate recognition by the *Conus* carboxylase. The γ-glutamyl recognition sequence was originally defined for the mammalian enzyme (3, 56–58). Cloning of the cDNA encoding the precursor form of conantokin-G (14) demonstrated that the propeptide sequence, specifically between residues −20 and −1 directs carboxylase interaction with the peptide substrate. A comparison of the propeptide sequence of conantokin-G to the Gla-containing ϵ-TxIX from *C. textile* revealed regions of homology and a common pattern of hydrophobic residues within the C-terminal propeptide regions (15). However, the propeptide of a third Gla-containing conotoxin, tx9a, isolated from *C. textile,* did not show any obvious sequence similarity to the other two *Conus* γ-carboxylation recognition sequences (48). Thus, a binding site for the *Conus* carboxylase has been difficult to identify. The non-homologous C-terminal portion of glacontryphan-M is rich in basic residues compared with other contryphan propeptide sequences. It is thus tempting to speculate that these basic residues make up part of the *Conus* γ-glutamyl recognition site.

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REFERENCES

1. Stenflo, J., and Suttie, J. W. (1977) *Annu. Rev. Biochem.* **46,** 157–172
2. Suttie, J. W. (1985) *Annu. Rev. Biochem.* **54,** 459–477
3. Furie, B., Bouchard, B. A., and Furie, B. A. (1999) *Blood* **93,** 1798–1808
4. Stenflo, J. (1999) *Crit. Rev. Eukaryot. Gene. Expr.* **9,** 59–88
5. McIntosh, J. M., Olivera, B. M., Cruz, L. J., and Gray, W. R. (1984) *J. Biol. Chem.* **259,** 14343–14346
6. Olivera, B. M., Rivier, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., Mena, E. E., Woodward, S. R., Hillyard, D. R., and Cruz, L. J. (1990) *Science* **20,** 257–263
7. Hauschka, P. V., Mullen, E. A., Hintsch, G., and Jazwinski, S. (1988) in *Current Advances in Vitamin K Research* (Suttie, J. W., ed) pp. 237–243, Elsevier, New York
8. Begley, G. S., Furie, B. C., Czerwiec, E., Taylor, K. L., Furie, G. L., Bronstein, L., Stenflo, J., and Furie, B. (2000) *J. Biol. Chem.* **275,** 356245–336249
9. Bandyopadhyay, P. K., Garrett, J. E., Shetty, R. P., Keate, T., Walker, C. S., and Olivera, B. M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 1264–1269
10. Czerwiec, E., Begley, G. S., Bronstein, M., Stenflo, J., Taylor, K., Furie, B. C., and Furie, B. (2002) *Eur. J. Biochem.* **269,** 6162–6172
11. Tao, L., Yang, C-T., Jin, D., and Stafford, W. (2000) *J. Biol. Chem.* **275,** 18291–18296
12. Walker, C. S., Shetty, R. P., Clark, K., Kazuko, S. G., Letsou, A., Olivera, B. M., and Bandyopadhyay, P. K. (2001) *J. Biol. Chem.* **276,** 7769–7774
13. Stanley, T. B., Stafford, D. W., Olivera, B. M., and Bandyopadhyay, P. K., (1997) *FEBS Lett.* **407,** 85–88
14. Bandyopadhyay, P. K., Colledge, C. J., Walker, C. S., Zhou, L., Hillyard, D. R., and Olivera, B. M. (1998) *J. Biol. Chem.* **273,** 5447–5450
15. Bush, K., Stenflo, J., Roth, D. A., Czerwiec, E., Harrist, A., Begley, G. S., Furie, B. C., and Furie, B. (1999) *Biochemistry* **38,** 14660–14666
16. Haack, J. A., Rivier, J., Parks, T. N., Mena, E. E., Cruz, L. J., and Olivera, B. M. (1990) *J. Biol. Chem.* **265,** 6025–6029
17. Fainzilber, M., Gordon, D., Hasson, A., Spira, M. E., and Zlotkin, E. (1991) *Eur. J. Biochem.* **202,** 589–595
18. Nakamura, T., Yu, Z., Fainzilber, M., and Burlingame, A. L. (1996) *Protein Sci.* **5,** 524–530
19. Fainzilber, M., Nakamura, T., Lodder, J. C., Zlotkin, E., Kits, K. S., and Burlingame, A. L. (1998) *Biochemistry* **37,** 1470–1477
20. Rigby, A. C., Lucas-Meunier, E., Kalume, D. E., Czerwiec, E., Hambe, B., Dahlqvist, I., Fossier, P., Baux, G., Roepstorff, P., Baleja, J. D., Furie, B. C., Furie, B., and Stenflo, J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5758–5763
21. Kalume, D. E., Stenflo, J., Czerwiec, E., Hambe, B., Furie, B. C., Furie, B., and Roepstorff, P. (2000) *J. Mass Spectr.* **35,** 145–156
22. Prorok, M., Warder, S. E., Blandl, T., and Castellino, F. J. (1996) *Biochemistry* **35,** 16528–16534
23. Rigby, A. C., Baleja, J. D., Furie, B.C., and Furie, B. (1997) *Biochemistry* **36,** 6906–6914
24. Rigby, A., Baleja, L., Li, L., Pedersen, L. G., Furie, B. C., and Furie, B. (1997) *Biochemistry* **36,** 15677–15684
25. Skjaerbaek, N., Nielsen, K. J., Lewis, R. J., Alewood, P., and Craik, D. J. (1997) *J. Biol. Chem.* **272,** 2291–2299
26. Chen, Z., Blandl, T., Prorok, M., Warder, S. E., Li, L., Zhu, Y., Pedersen, L. G.,

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Ni, F., and Castellino, F. J. (1998) *J. Biol. Chem.* **26,** 16248–16258
27. Prorok, M., and Castellino, F. J. (1998) *J. Biol. Chem.* **273,** 19573–19578
28. Blandl, T., Warder, S. E., Prorok, M., and Castellino, F. J. (2000) *FEBS Lett.* **470,** 139–146
29. Chandler, P., Pennington, M., Maccecchini, M. L., Nashed, N. T., and Skolnick, P. (1993) *J. Biol. Chem.* **268,** 17173–17178
30. Zhou, L. M., Szendrei, G. I., Fossom, L. H., Maccecchini, M. L., Skolnick, P., and Otvos, L., Jr. (1996) *Neurochemistry* **66,** 620–628
31. Jimenez, E. C., Craig, A. G., Watkins, M., Hillyard, D. R., Gray, W. R., Gulyas, J., Rivier, J. E., Cruz, L. J., and Olivera, B. M. (1997) *Biochemistry* **36,** 989–994
32. Jacobsen, R., Jimenez, E. C., Grilley, M., Watkins, M., Hillyard, D., Cruz, L. J., and Olivera, B. M. (1998) *J. Pept. Res.* **51,** 173–179
33. Jimenez, E. C., Watkins, M., Juszczak, L. J., Cruz, L. J., and Olivera, B. M. (2001) *Toxicon* **39,** 803–808
34. Massilia, G. R., Schininá, M. E., Ascenzi, P., and Polticelli, F. (2001) *Biochem. Biophys. Res. Commun.* **288,** 908–913
35. Jimenez, E. C., Olivera, B. M., Gray, W. R., and Cruz, L. J. (1996) *J. Biol. Chem.* **271,** 28002–28005
36. Jacobsen, R., Jimenez, E. C., De la Cruz, R. G. C., Gray, W. R., Cruz, L. J., and Olivera, B. M. (1999) *J. Pept. Res.* **54,** 93–99
37. Massilia, G. R., Eliseo, T., Grolleau, F., Lapied, B., Barbier, J., Bournaud, R., Molgó, J., Cicero, D. O., Paci, M., Schininá, M. E., Ascenzi, P., and Poltizelli F. (2003) *Biochem. Biophys. Res. Commun.* **303,** 238–246
38. Burgoyne, R. D., and Morgan, A. (1993) *Biochem. J.* **293,** 305–316
39. Barg, S., Ma, X., Eliasson, L., Galvanovskis, J., Göpel, S. O., Obermuller, S., Platzer, J., Renström, E., Trus, M., Atlas, D., Striessnig, J., and Rorsman, P. (2001) *Biophys. J.* **81,** 3308–3323
40. Stenflo, J., Fernlund, P., Egan, W., and Roepstorff, P. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 2730–2733
41. Fernlund, P., and Stenflo, J. (1975) *J. Biol. Chem.* **250,** 6125–6133
42. Cairns, J. R., Williamson, M. K., and Price, P. A. (1991) *Anal. Biochem.* **199,** 93–97
43. Nordenman, B., Danielsson, A., and Björk, I. (1978) *Eur. J. Biochem.* **90,** 1–6
44. Eliasson, L., Ma, X., Renström, E., Barg, S., Berggren, P. O., Galvanovskis, J., Gromada, J., Jing, X., Lundquist, I., Salehi, A., Sewing, S., and Rorsman, P. (2003) *J. Gen. Physiol.* **121,** 181–197
45. Barg, S., Galvanovskis, J., Göpel, S. O., Rorsman, P., and Eliasson, L. (2000) *Diabetes* **49,** 1500–1510
46. Rae, J., Cooper, K., Gates, P., and Watsky, M. (1991) *J. Neurosci. Methods* **37,** 15–26
47. Grant, M. A., Hansson, K., Furie, B. C., Furie, B., Stenflo, J., and Rigby, A. (2004) *J. Biol. Chem.* **279,** 32464–32473
48. Lirazan, M. B., Hooper, D., Corpuz, G. P., Ramilo, C. A., Bandyopadhyay, P., Cruz, L. J., and Olivera, B. M. (2000) *Biochemistry* **39,** 1583–1588
49. Schulla, V., Renström, E., Feil, R., Feil, S., Franklin, I., Gjinovci, A., Jing, X. J., Laux, D., Lundquist, I., Magnuson, M. A., Obermüller, S., Olofsson, C. S., Salehi, A., Wendt, A., Klugbauer, N., Wollheim, C. B., Rorsman, P., and Hofmann, F. (2003) *EMBO J.* **22,** 3844–3854
50. Fainzilber, M., Lodder, J. C., van der Schors, R. C., Li, K. W., Yu, Z., Burlingame A. L., Geraerts, W. P. M., and Kits, K. S. (1996) *Biochemistry* **35,** 8748–8752
51. Kreil, G (1997) *Annu. Rev. Biochem.* **66,** 337–345
52. Miles, L. A., Dy, C. Y., Nielsen, J., Barnham, K.J., Hinds, M. G., Olivera, B. M., Bulaj, G., and Norton, R. S. (2002) *J. Biol. Chem.* **277,** 43033–43040
53. Bokvist, K., Eliasson, L., Ämmala, C., Renström, E., and Rorsman, P. (1995) *EMBO J.* **14,** 50–57
54. Wiser, O., Trus, M., Hernandez, A., Renström, E., Barg, S., Rorsman, P., and Atlas, D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 248–253
55. Caterall, W. A. (2000) *Annu. Rev. Dev. Biol.* **16,** 521–555
56. Jorgensen, M. J., Cantor, A. B., Furie, B. C., Brown, C. L., Shoemaker, C. B., and Furie B. (1987) *Cell* **48,** 185–191
57. Jorgensen, M. J., Cantor, A. B., Furie, B. C., and Furie, B. (1987) *J. Biol. Chem.* **262,** 6729–6734
58. Pan, L. C., and Price, P. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 6109–6113

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