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Specialized insulin is used for chemical warfare by fish-hunting cone snails

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More than 100 species of venomous cone snails (genus *Conus*) are highly effective predators of fish. The vast majority of venom components identified and functionally characterized to date are neurotoxins specifically targeted to receptors, ion channels, and transporters in the nervous system of prey, predators, or competitors. Here we describe a venom component targeting energy metabolism, a radically different mechanism. Two fish-hunting cone snails, *Conus geographus* and *Conus tulipa*, have evolved specialized insulins that are expressed as major components of their venoms. These insulins are distinctive in having much greater similarity to fish insulins than to the molluscan hormone and are unique in that posttranslational modifications characteristic of conotoxins (hydroxyproline, γ-carboxyglutamate) are present. When injected into fish, the venom insulin elicits hypoglycemic shock, a condition characterized by dangerously low blood glucose. Our evidence suggests that insulin is specifically used as a weapon for prey capture by a subset of fish-hunting cone snails that use a net strategy to capture prey. Insulin appears to be a component of the nirvana cabal, a toxin combination in these venoms that is released into the water to disorient schools of small fish, making them easier to engulf with the snail's distended false mouth, which functions as a net. If an entire school of fish simultaneously experiences hypoglycemic shock, this should directly facilitate capture by the predatory snail.

insulin shock | cone snails | conotoxins | nirvana cabal | venom

The venoms of predatory marine cone snails are remarkably potent and diverse (1). Most bioactive venom components are small disulfide-rich peptides, termed conotoxins (1), that target specific receptors and ion channel subtypes located in the prey's nervous system (2, 3). Here, we provide evidence for a specialized insulin in the venom of *Conus geographus* that is part of the chemical arsenal used by the snail for capturing prey. This finding significantly extends known molecular mechanisms of envenomation beyond conventional neurotoxins.

Unlike other fish-hunting cone snails that inject venom as they tether the prey, *C. geographus* engulfs its fish prey with its highly distended false mouth before venom injection (1). It has been suggested that *C. geographus* releases specialized toxins, called the "nirvana cabal," into the water to suppress the sensory circuitry of schools of small fish prey (4). Our data strongly suggest that the specialized *C. geographus* insulin we characterized, Con-Ins G1, forms part of this venom cabal.

Vertebrate insulin, synthesized in pancreatic β cells, is the key hormone regulator of carbohydrate and fat metabolism (5); in the brain, it functions as a neuromodulator of energy homeostasis and cognition (6). Insulin is initially synthesized as a precursor comprising three regions (A, B, and C) (7), from which proteolytic cleavage of the C peptide in the Golgi releases the mature insulin heterodimer with an A and B chain connected by two disulfide bonds. The A chain contains an additional intramolecular disulfide. The primary sequence and arrangement of cysteines that form disulfides are highly conserved in all vertebrates (5). In contrast, invertebrate insulin family members are more variable and serve in neuronal signaling, memory, reproduction, growth, and metabolism (8, 9). In molluscs, insulins are primarily expressed in neuroendocrine cells, including neurons and cerebral ganglia (9, 10). Molluscan and most other invertebrate insulins differ from the vertebrate hormone in containing two additional cysteines (one in each chain) that are assumed to form an additional disulfide between the A and B chain (9). Moreover, the mature molluscan insulin chains are generally larger than vertebrate insulins. Thus, sea hare (*Aplysia californica*) insulin (AI) has a molecular mass of 9,146 Da (10) (compared with 5,808 Da for human insulin).

The venom gland of cone snails is highly specialized for conotoxin biosynthesis and secretion (11). Our discovery that an insulin is expressed in the venom gland of *C. geographus* at levels

Significance

The discovery and characterization of insulin, a key hormone of energy metabolism, provided a life-saving drug for diabetics. We show that insulin can be subverted for nefarious biological purposes: Venomous cone snails use specialized insulins to elicit hypoglycemic shock, facilitating capture of their fish prey. This finding extends our understanding of the chemical and functional diversity of venom components, such that the snail's arsenal includes a diverse set of neurotoxins that alters neuronal circuitry, as well as components that override glucose homeostasis. The highly expressed venom insulins are distinct from molluscan insulins and exhibit remarkable similarity to fish insulins. They are the smallest of all insulins characterized from any source, potentially providing new insights into structure-function elements of insulin action.

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. KP268599–KP268619).

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comparable to conotoxins was unexpected. When injected into fish this insulin significantly lowers blood glucose levels, and direct application into the water column significantly reduces locomotor activity. We show that this peptide has unusual features, including posttranslational modifications unprecedented in insulin but often found in conotoxins.

Results

Identification of a Fish-Like Insulin in the Venom Gland of *C. geographus*. Analysis of next-generation sequencing of the venom gland transcriptome of *C. geographus* (12) led to the identification of two transcripts with high sequence similarity to insulin. These transcripts were named *C. geographus* insulins 1 and 2 (Con-Ins G1 and Con-Ins G2). Like other insulins, Con-Ins G1 and G2 precursors consist of four distinct regions: an N-terminal signal peptide and a B and A chain separated by one or more C peptides (Fig. 1). Based on this precursor organization, it can be predicted that oxidative folding and proteolytic processing of the two *C. geographus* transcripts yields mature heterodimers that consist of A and B chains connected by disulfide bonds.

The predicted mature Con-Ins G1 shares high similarity with vertebrate insulin, whereas Con-Ins G2 more closely resembles endogenous molluscan insulins. These differences are most obvious in the cysteine pattern and the lengths of the predicted A and B chains. Like vertebrate insulins, Con-Ins G1 has relatively short chains and contains four cysteines in the A and two in the B chain. In contrast, like most other invertebrate insulins (9), Con-Ins G2 contains two additional cysteines, one in each chain, and has longer A and B chains compared with vertebrate insulins.

Con-Ins G1 shows striking sequence similarity to insulins from fish, the prey of *C. geographus*. This similarity is most apparent within the A chain for which 90% of amino acids are similar between this peptide and insulins from zebrafish (*Danio rerio*) and the white sucker, *Catostomus commersonii* (Fig. 2). In particular, the N-terminal half of Con-Ins G1 is almost identical to the fish peptides. Thus, Con-Ins G1 is more similar to fish insulins than to any other vertebrate insulin. Similarities within the B chains are lower, ranging from 68% to 73%.

Although Con-Ins G2 shares a cysteine pattern with molluscan insulin, similarities at the amino acid level are relatively low, ranging from 50% to 71% compared with the A and B chains of the sea hare *Aplysia californica* and the great pond snail *Lymnea stagnalis* (Fig. 2). This finding is consistent with the observation that invertebrate insulin sequences are less conserved than their vertebrate counterparts (9).

In molluscs, insulins are typically expressed in neuroendocrine tissues, including ganglia and neurons (9), so the identification of insulin sequences in the venom gland of *C. geographus* was unexpected. Furthermore, *C. geographus* insulins represented the most highly expressed non–conotoxin-encoding genes, with expression levels comparable to established venom peptides. (Table 1) (12). The venom gland of cone snails is a long, heterogeneous

Fig. 1. Identification of two insulin transcripts in the venom gland of *C. geographus*: Con-Ins G1 and G2. The top panel shows the typical precursor organization and disulfide connectivites of insulin (the additional disulfide found in molluscs is shown in light gray). *C. geographus* sequences are depicted below and follow the same color coding. The signal peptide was determined using SignalP software (29). The location of the A and B chains were predicted based on comparison with known insulin sequences.

Fig. 2. Similarity in cysteine arrangement, chain lengths, and amino acid residues between the A and B chains of Con-Ins G1 and fish insulin, and Con-Ins G2 and the molluscan peptide. (*A*) Comparative alignment between Con-Ins G1 and insulins from the zebrafish *Danio rerio* and the white sucker *Catostomus commersonii*. (*B*) The molluscan-like peptide Con-Ins G2 was aligned with insulin sequences from the sea hare *Aplysia californica* (AI) and the giant pond snail *Lymnea stagnalis* (MIP1). Chain lengths and number of cysteines are provided following the sequence. Cysteines are highlighted in yellow. Identical amino acids are shown in bold. Amino acid conservation is denoted by an asterisk (*). Full stops (.) and colons (:) represent a low and high degree of similarity, respectively.

organ that is highly specialized for the biosynthesis and secretion of conotoxins (11). Even though toxin expression is found across the entire length of the venom gland, expression of Con-Ins G1 was almost exclusively found in the distal region of the venom gland closest to the injection apparatus (99.9% of all RNA-Seq reads that mapped to Con-Ins G1 were found in this region; Table 1). In contrast, read numbers for Con-Ins G2 were very low in this region (0.9%) but high in the proximal section (49%).

The Type of Insulin Expressed in the Venom Gland Correlates with Feeding Preference. According to their prey preference, cone snails can be grouped into fish-, mollusc-, and worm-hunting snails. Several representatives of each feeding type were selected, and insulin expression was investigated by targeted RT-PCR and mining of cDNA libraries and transcriptome data. The presence of Con-Ins G1 and G2 in the venom gland of *C. geographus* was confirmed using RT-PCR combined with PCR cloning and Illumina sequencing. An additional sequence, Con-Ins G3, was also identified, albeit at lower expression levels (283 total reads retrieved from transcriptome data). Like Con-Ins G1, Con-Ins G3 contained the vertebrate-like arrangement of cysteines (Fig. S1) and was expressed exclusively in the distal region of the venom gland (100% of all reads). An identical sequence was recently reported in *C. geographus* as "new superfamily 2 conotoxin G121" but not recognized as an insulin (13). Several variants of Con-Ins G1, G2, and G3 were identified by RT-PCR, a phenomenon that has also been described for other cone snail venom peptides (12) (Fig. S1). All sequences obtained by PCR were also retrieved from the venom gland transcriptome database.

Using the same methodology, three insulin sequences were identified from the venom gland of *Conus tulipa*, another member of the Gastridium clade and the closest known relative of *C. geographus* (14). These sequences were named Con-Ins T1, T2, and T3. All three exhibit the cysteine arrangement of vertebrate insulin and share high overall sequence similarity with Con-Ins G1 and therefore fish insulins (Fig. S2). Sequence identities to Con-Ins G1 across the entire length of the prepropeptide range

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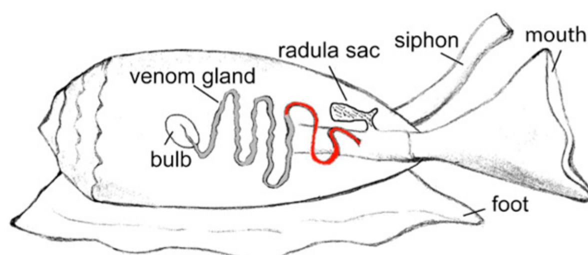
Table 1. Expression levels and distribution of *C. geographus* insulins across the length of the venom gland

	Total reads	Distribution of reads in four sections of the *C. geographus* venom gland (%)			
		1 (proximal)	2 (proximal central)	3 (distal central)	4 (distal)
Insulins					
Con-Ins G1	1,242	0	0.1	0	99.9
Con-Ins G2	1,120	49.5	30.8	18.8	0.9
Nirvana cabal toxins					
Conantokin G	38,854	0.4	0.1	36.9	62.6
Conopressin G	310	4.5	10.2	9.1	76.2
Contulakin G	899	0.1	0.1	21.1	78.7
Motor cabal toxins					
μ-GIIIB	114,509	42.5	43.0	14.4	0.2
ω-GVIA	3,655	74.0	17.3	7.2	1.5
α-GII	19,059	47.5	36.1	15.0	1.4



The transcriptomes of four regions of the venom gland were sequenced by 454 RNA-Seq and differential expression of insulin transcripts and selected conotoxins was determined by mapping all reads to the transcripts of interest. The (distal) region of the venom gland is highlighted in red in the anatomical schematic. Read numbers for transcripts with highest expression in this region are also shown in red.

from 83% to 87%. The predicted mature peptide of the most similar transcript, Con-Ins T1, differs by only one conservative substitution in the A chain (Tyr-19 to Phe) and five substitutions in the B chain, of which three are conservative (Fig. S2). No sequences resembling endogenous molluscan insulins were found in the venom gland of *C. tulipa*, despite deep sequencing of PCR products (a total of 274,520, 187,930, and 132,975 reads were obtained for Con-Ins T1, T2, and T3, respectively).

Five additional fish-hunting cone snails were examined by RT-PCR using the same oligonucleotides and PCR conditions used for *C. geographus* and *C. tulipa* (Table S1). Unlike *C. geographus* and *C. tulipa*, which use a "net-hunting" strategy for prey capture, these species belong to the "hook and line hunters" (1). RT-PCR did not identify insulin transcripts in any of the five tested species. For two of these snails, *C. bullatus* and *C. consors*, the absence of insulin transcripts was also confirmed by mining next-generation transcriptome data (15, 16) [*C. consors* transcriptome data have not been made publically available, but Violette et al. (16) do not report any insulin expression in the venom gland of this species].

The venom glands of two mollusc and four worm-hunting cone snails were also investigated; all expressed molluscan-like insulins, but not the fish-like peptides (Table S1 and Fig. S1). As observed for Con-Ins G2, these transcripts were characterized by an additional cysteine residue in each chain and longer predicted A and B chains. Sequence diversity ranged from one transcript for *Conus marmoreus*, *Conus textile*, and *Conus memiae* to four in *Conus floridulus*. These findings suggest that insulin expression in the venom gland of a particular species can be correlated with its feeding strategy. Molluscan-like insulins are broadly distributed among snail and worm hunters, whereas the expression of fish-like insulins appears to be limited to a subset of fish hunters.

Con-Ins G1: High Abundance and Novel Posttranslational Modifications. To determine whether *C. geographus* insulin transcripts were translated and posttranslationally processed into mature insulins, the venom of this species was interrogated by MS. Mature peptides derived from the Con-Ins G1 precursor but not G2 were identified by MS sequencing performed on reduced and alkylated (RA) venom using a high-resolution Orbitrap Elite instrument.

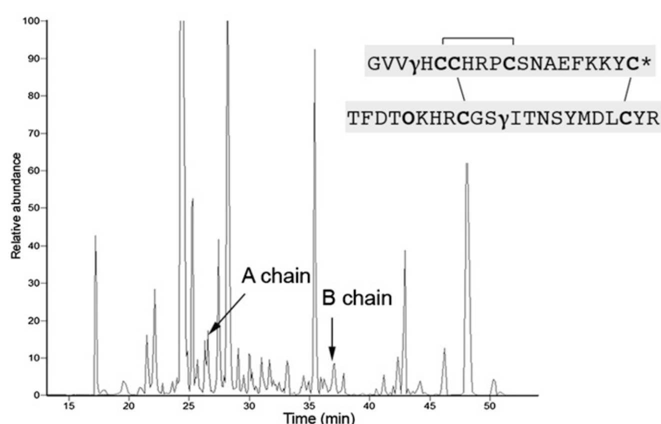


Fig. 3. MS sequencing of Con-Ins G1 in the venom of *C. geographus* identifies the A and B chain and several modifications unusual for insulins. (*A*) Venom was reduced and alkylated and subjected to reverse-phased chromatography and tandem MS sequencing on a high-resolution Orbitrap Elite mass spectrometer. Tandem MS data are provided in Fig. S3. Posttranslational modifications are provided in their single letter code. γ, γ-carboxylated glutamate; O, hydroxyproline, *, C-terminal amidation. (*Right*) The resulting Con-Ins G1 heterodimer. Cysteines and modified residues are shown in bold.

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Tandem MS identified the individual A and B chains of Con-Ins G1, demonstrating that cleavage of the precursor peptide at basic or dibasic sites liberates an A and B chain, as observed for other insulins (Fig. 3; MS/MS spectra in Fig. S3). Con-Ins G1 carries several posttranslational modifications that are unprecedented in insulins but commonly observed in other cone snail venom peptides (17). Glu4 of the A chain, one of the most conserved amino acids in vertebrate insulin (18), is modified to γ-carboxylated glutamic acid (Gla or γ). Another Gla modification (Gla12) was also identified in the B chain. Moreover, Pro5 in the A chain is modified to hydroxyproline (Hyp5), and the C terminus of this chain is amidated (Fig. 3).

MS sequencing further revealed the presence of several variants of Con-Ins G1 in fractionated venom (Fig. S4). Modifications in these variants include Gla15 and Hyp10 in the A chain, Hyp5 in the B chain, and alternative proteolytic cleavage at the C terminus of the B chain. The presence of multiple variants deriving from the same peptide precursor has recently been described for several conotoxins (16, 19) but is exceptional for insulins. The other vertebrate-like insulin, Con-Ins G3, was also identified by MS sequencing (Fig. S5). Similar to Con-Ins G1, this peptide is modified, and several variants could be identified in the venom (Fig. S4).

Fractionated *C. geographus* venom was analyzed by high-resolution LC-MS and searched for masses corresponding to the intact insulin dimers. A heterodimer that consisted of the originally sequenced A and B chains (Fig. 3) was not identified, but MS sequencing successfully identified one of the variants that contained an additional γ-carboxylated glutamic acid in the A chain (Gla15) and did not have the N-terminal arginine in the B chain (Fig. S6*A*). The molecular mass of this variant was 5,030.06 Da, consistent with the presence of three disulfide bonds. A second variant containing an additional hydroxyproline (Hyp10) in the A chain (5,046.05 Da; Fig. S6*B*) and a third variant that had no hydroxylated prolines in either chain were also identified (5,014.05 Da; Fig. S6*C*). Fractions were reduced and alkylated, and sequences were confirmed by tandem MS sequencing (Fig. S5). These data clearly show that Con-Ins G1 forms a heterodimer consisting of A and B chains that are linked by disulfide bonds. The intact heterodimer of the other vertebrate-like peptide, Con-Ins G3, was also identified by MS sequencing. Intact Con-Ins G3 has a molecular mass of 4,817.10 Da, consistent with the presence of three disulfide bonds (Fig. S6*D*). With a total length of 43 amino acids, to our knowledge, native Con-Ins G1 and G3 are the shortest native insulins reported to date (human, zebrafish, and *Aplysia* insulins are 51, 50, and 80 residues long, respectively).

Venom fractionation and sequencing further confirmed that Con-Ins G1 and its variants are abundant peptides in the venom of *C. geographus*. The venom fraction containing Con-Ins G1 was 1 of 25 collected fractions and predominantly consisted of venom insulins.

Con-Ins G1 Was Successfully Synthesized Using a Selenocysteine-Based Strategy. To obtain sufficient material to investigate its bioactivity in fish, Con-Ins G1 was chemically synthesized. Since the first chemical synthesis in 1963 (20), insulin has remained challenging to synthesize with the correct intra- and intermolecular disulfide bonds. A strategy incorporating a pair of selenocysteines in the A chain was used to synthesize Con-Ins G1. Replacement of one pair of cysteines with selenocysteines (Sec) has been used for the synthesis of various bioactive peptides, including conotoxins (21). Diselenide bond-containing peptide analogs have similar biological activities to their native peptides and, in some cases, even improved potency or selectivity (21). Due to the lower redox potential, diselenide bond formation is favored over the disulfide bond formation under acidic conditions (21). This Cys-to-Sec replacement strategy was combined with

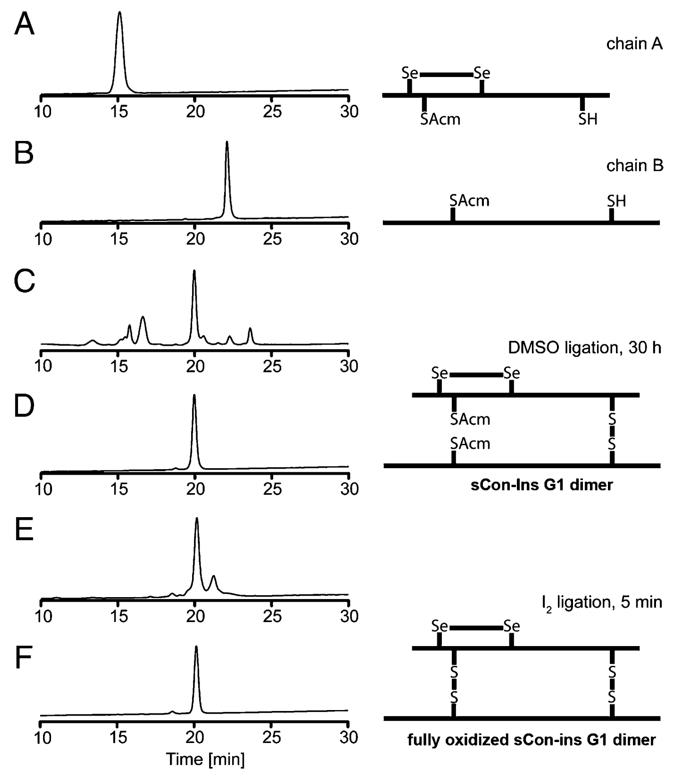

Fig. 4. Synthesis of sCon-Ins G1. (*A*) Purified chain A with the intramolecular Sec-Sec bridge formed and Cys-7 protected with the Acm group. (*B*) Purified chain B with Cys-9 protected with the Acm group. (*C*) sCon-Ins G1 heterodimer formation by treatment with 20% DMSO for 30 h in 0.1 M Tris·HCl containing 1 mM EDTA, pH 7.5. (*D*) Purified sCon-Ins G1 heterodimer. (*E*) Oxidation of remaining disulfide bond of the heterodimer of sCon-Ins G1 by treatment with I_2 in the mixture of acetonitrile, water, and TFA at 200 μM for 5 min. (*F*) Purified final sCon-Ins G1.

orthogonal protection of the remaining two pairs of cysteines to sequentially form intra- and intermolecular disulfide bridges, as shown in Fig. 4. Cys6 and Cys11 of chain A were replaced with Sec residues, which formed an intradiselenide bridge after peptide cleavage and reduction (21, 22) (Fig. 4*A*). Cys20A and Cys21B of the respective chains [synthesized with a trityl (Trt) protecting group] were used to form the first intermolecular disulfide bridge on DMSO treatment (Fig. 4*C*). Cys7A and Cys9B, bearing acetaminomethyl (Acm) groups, were subjected to iodine oxidation to remove Acm protection and simultaneously form the second disulfide bridge (Fig. 4*E*). The two-step ligation protocol led to the final purified product, synthetic selenocysteine-Con-Ins G1 (sCon-Ins G1), in a total yield of 10.5%, based on the starting amount of purified chain A. RP-HPLC confirmed the purity (95%), and electrospray ionization MS sequencing confirmed the correct identity of the product (Fig. S7). To our knowledge, this is the first time a selenocysteine-based strategy has been applied to the synthesis of insulin.

sCon-Ins G1 Lowers Blood Glucose and Alters Swimming Activity in Fish. The streptozotocin (STZ)-induced model of hyperglycemia was used to assess whether sCon-Ins G1 could effectively lower blood glucose levels in a model prey: adult zebrafish. Animals were first rendered hyperglycemic through i.p. injection of the β-cell poison STZ (1.5 g/kg) (23), and the effect of subsequent injection of sCon-Ins G1 was examined. Following STZ treatment, blood glucose levels were significantly elevated from 65.9 ± 4.75 (n = 11) to 273.0 ± 34.08 mg/dL (n = 5, P < 0.01; Fig. 5*A*). Administration of sCon-Ins G1 at 65 ng peptide/g body weight

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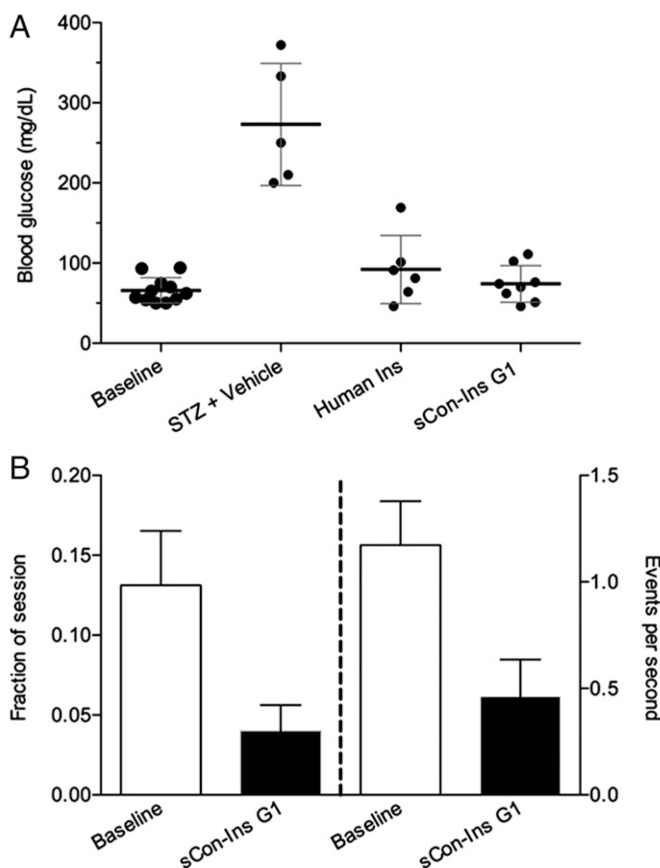

Fig. 5. sCon-Ins G1 lowers blood glucose and disrupts swimming behavior in zebrafish. (*A*) Insulin activity was determined using the streptozotocin (STZ)-induced model of hyperglycemia. Injection of 1.5 g/kg STZ into adult zebrafish caused hyperglycemia as evident by a significant increase of blood glucose (n = 5 fish, P = 0.003) compared with controls (n = 11). Hyperglycemia was successfully reversed on administration of 65 ng/g sCon-Ins G1 (n = 8, P = 0.003) and human insulin (65 ng/g, n = 6, P < 0.003). Data were analyzed in Prism Graphpad software (version 6.0) using unpaired *t* tests with Welch's correction. Plotted dots represent individual fish. Bold lines and error bars represent means ± SD. (*B*) Application of sCon-Ins G1 into the water column (25 nmol/mL) significantly reduced overall locomotor activity, observed as a significant decrease in the percentage of time spent swimming (P = 0.038, left *y* axis) and movement frequency (P = 0.003, right *y* axis) compared with baseline values for the same animals. Values represent means ± SEM (n = 3).

significantly lowered blood glucose to 74.0 ± 8.051 mg/dL (n = 8, P < 0.01). The ability to effectively lower blood glucose was similar to human insulin (92.0 ± 17.35 mg/d, n = 6, P < 0.01).

To determine whether sCon-Ins G1 elicits an effect when applied to the water column, the spontaneous swimming behavior of fish larvae (7 d after fertilization, n = 3) was monitored before (baseline) and following application of 25 nmol/mL peptide. Administration of sCon-Ins G1 reduced overall locomotor activity, observed as a significant decrease in the percentage of time spent swimming (P = 0.038) and movement frequency (P = 0.003) compared with baseline values for the same animals (Fig. 5*B* and Movie S1). It has previously been reported that externally applied human insulin can induce insulin shock in fish via direct absorption through the gills into the bloodstream (24). Our findings suggest that sCon-Ins G1 also efficiently crosses the endothelium of the gill plexus.

Discussion

Insulin is an essential hormone, but overdoses cause hypoglycemia, and in extreme cases, fatality. The deliberate use of insulin for nefarious purposes is both the material of fiction and regularly rumored to occur in real life. In the famous von Bülow case, an alleged overdose of insulin became the basis for a sensational criminal trial. Remarkably, evolution has developed a parallel story line, with the normal physiological function of insulin being subverted by a lineage of cone snails as part of their strategy to overcome their fish prey. Our results establish that species in the Gastridium clade, one of five to six clades of fish-hunting cone snails, use insulin as an offensive weapon, part of the chemical armamentarium evolved for maximizing the probability of prey capture.

Cone snails, like all molluscs, have endogenous insulin, and we identified a sequence resembling endogenous molluscan insulins in the venom glands of *C. geographus* and several vermivorous and molluscivorous species. These sequences differ from fish insulins in highly distinctive ways, notably the presence of an additional pair of cysteines. We demonstrate that cone snails in the Gastridium clade have, in addition to molluscan type insulin, a unique class of insulins that is expressed at very high levels in their venom. These specialized insulins share much greater similarity to the vertebrate insulin found in fish, the prey of these snails. We directly demonstrate that Con-Ins G1 is not only bioactive in fish, but, when this insulin is applied to the water, the fish become hypoactive.

Larger cone snails in the Gastridium clade, including *C. geographus* and *C. tulipa* use a net strategy for prey capture. It has been suggested that in the wild, these snails primarily stalk schools of small fish hiding in reef crevices at night. By extruding a subset of venom components, known as the nirvana cabal, as they approach the school of fish, they are able to elicit a state of sensory deprivation and general hypoactivity (4). Suggested members of this nirvana cabal include conantokin-G, a selective antagonist of the NMDA receptor (25); contulakin-G, a neurotensin receptor agonist (26); and Lys-conopressin-G, an agonist of the vasopressin receptor (27). Like Con-Ins G1, these peptides are most highly expressed in the distal section of the long and convoluted venom gland, closest to the injection apparatus (Table 1). In contrast, venom components that cause irreversible neuromuscular block (motor cabal toxins) (4), including the nicotinic acetylcholine antagonist α-GII and the sodium channel blocker μ-GIIIB, display very low expression levels in this region.

The present evidence shows that the nirvana cabal targets not only the circuitry of the fish nervous system but also energy metabolism because Con-Ins G1, when released, results in hypoglycemic shock in the fish. The symptoms of low blood glucose, which include weakness and neuronal dysfunction, are characteristic of physiological impairments induced by the nirvana cabal (4). Three additional fish-like insulin sequences were also identified in the venom gland of *C. tulipa*, a close relative of *C. geographus*. However, specialized insulins that are venom components in these fish-hunting species are absent in venoms of other fish hunters that do not use a net strategy, but instead harpoon their prey. Moreover, cone snails that prey on snails and worms do not express the fish-like sequence but insulins that are similar to endogenous molluscan and worm insulins, suggesting that these insulins also play a role in prey capture.

High expression levels of the molluscan-like insulin Con-Ins G2 in the proximal region of the venom gland of *C. geographus* are consistent with recent observations on the potential role of this region for the expression of toxins destined for defense rather than predation (13).

To our knowledge, insulin has not been reported in any other animal venom. However, venom components that target the prey's metabolism and, more specifically, glucose homeostasis, appear to be more widespread. Although mechanistically different to cone snail venom insulins, exendin-4 isolated from the saliva venom of the gila monster *Heloderma suspectum* likely elicits a similar physiological response. Exendin-4 is a potent agonist of the glucagon-like peptide receptor and promotes the

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secretion of endogenous insulin in the prey (28). Exendin-4 has been developed as a commercial drug (exenatide) and effectively treats diabetes mellitus, highlighting the therapeutic potential of venom hormone mimics (28).

Evolving insulin to be used as a pharmacological weapon presents a novel and unexpected type of event in chemical biology. Many of the underlying mechanisms of the chemical interactions between animals are likely to be as surprising as the scenario detailed in this study. Our study reveals a class of insulins that have evolved to act rapidly and potently to cause severe hypoglycemia. It is therefore not so surprising that this is the shortest insulin yet discovered; at the molecular level, all regulatory aspects embedded in the insulin structure are nonessential details for this class of insulins that may be impervious to the subtle aspects of regulatory physiology. Their short length and posttranslational modifications may well reflect their streamlined physiological role in disrupting glucose homeostasis. Consequently, although normal vertebrate insulin signaling needs to be sensitive to downstream effects, these insulins may not be so constrained. A more comprehensive study of the structure and function of this special class of insulins, and a comparison with normal insulins, may provide insight into the structural elements of insulins, which are necessary for the more subtle regulation of these important hormones in normal physiology.

Materials and Methods

Detailed material and methods are provided in *SI Material and Methods*. Briefly, insulin transcripts were sequenced from cone snail venom glands by RT-PCR and/or next generation transcriptome sequencing. The two fish-like insulins, Con-Ins G1 and G3, were identified in the venom of *C. geographus* by reversed-phase chromatography combined with high-resolution mass spectrometry. Con-Ins G1 was chemically synthesized using a selenocysteine-based strategy and tested on zebrafish using the STZ-induced model of hyperglycemia and by direct application of the peptide into the water column.

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1. Olivera BM (1997) E.E. Just Lecture, 1996. Conus venom peptides, receptor and ion channel targets, and drug design: 50 million years of neuropharmacology. *Mol Biol Cell* 8(11):2101–2109.
2. Olivera BM, Teichert RW (2007) Diversity of the neurotoxic *Conus* peptides: A model for concerted pharmacological discovery. *Mol Interv* 7(5):251–260.
3. Terlau H, Olivera BM (2004) *Conus* venoms: A rich source of novel ion channel-targeted peptides. *Physiol Rev* 84(1):41–68.
4. Olivera BM (2002) *Conus* venom peptide: Reflections from the biology of clades and species. *Annu Rev Ecol Syst* 33:25–47.
5. Blumenthal S (2010) From insulin and insulin-like activity to the insulin superfamily of growth-promoting peptides: A 20th-century odyssey. *Perspect Biol Med* 53(4):491–508.
6. Gerozissis K (2003) Brain insulin: regulation, mechanisms of action and functions. *Cell Mol Neurobiol* 23(1):1–25.
7. Ward CW, Lawrence MC (2011) Landmarks in insulin research. *Front Endocrinol (Lausanne)* 2:76.
8. Wu Q, Brown MR (2006) Signaling and function of insulin-like peptides in insects. *Annu Rev Entomol* 51:1–24.
9. Smit AB, et al. (1998) Towards understanding the role of insulin in the brain: Lessons from insulin-related signaling systems in the invertebrate brain. *Prog Neurobiol* 54(1): 35–54.
10. Floyd PD, et al. (1999) Insulin prohormone processing, distribution, and relation to metabolism in Aplysia californica. *J Neurosci* 19(18):7732–7741.
11. Safavi-Hemami H, et al. (2014) Combined proteomic and transcriptomic interrogation of the venom gland of Conus geographus uncovers novel components and functional compartmentalization. *Mol Cell Proteomics* 13(4):938–953.
12. Hu H, Bandyopadhyay PK, Olivera BM, Yandell M (2012) Elucidation of the molecular envenomation strategy of the cone snail *Conus geographus* through transcriptome sequencing of its venom duct. *BMC Genomics* 13(284):284.
13. Dutertre S, et al. (2014) Evolution of separate predation- and defence-evoked venoms in carnivorous cone snails. *Nat Commun* 5:3521.
14. Puillandre N, Duda TF, Meyer C, Olivera BM, Bouchet P (2013) One, four or 100 genera? A new classification of the cone snails. *J Molluscan Studies* 80(4).
15. Hu H, Bandyopadhyay PK, Olivera BM, Yandell M (2011) Characterization of the *Conus bullatus* genome and its venom-duct transcriptome. *BMC Genomics* 12(1):60.
16. Violette A, et al. (2012) Large-scale discovery of conopeptides and conoproteins in the injectable venom of a fish-hunting cone snail using a combined proteomic and transcriptomic approach. *J Proteomics* 75(17):5215–5225.
17. Buczek O, Bulaj G, Olivera BM (2005) Conotoxins and the posttranslational modification of secreted gene products. *Cell Mol Life Sci* 62(24):3067–3079.
18. Mayer JP, Zhang F, DiMarchi RD (2007) Insulin structure and function. *Biopolymers* 88(5):687–713.
19. Tayo LL, Lu B, Cruz LJ, Yates JR, 3rd (2010) Proteomic analysis provides insights on venom processing in *Conus textile*. *J Proteome Res* 9(5):2292–2301.
20. Meienhofer J, et al. (1963) Synthese der Insulinketten und ihre Kombination zu Insulinaktiven Preaparaten. *Z Naturforsch B* 18:1120–1121.
21. Walewska A, et al. (2009) Integrated oxidative folding of cysteine/selenocysteine containing peptides: Improving chemical synthesis of conotoxins. *Angew Chem Int Ed Engl* 48(12):2221–2224.
22. Harris KM, Flemer S, Jr, Hondal RJ (2007) Studies on deprotection of cysteine and selenocysteine side-chain protecting groups. *J Pept Sci* 13(2):81–93.
23. Olsen AS, Sarras MP, Jr, Intine RV (2010) Limb regeneration is impaired in an adult zebrafish model of diabetes mellitus. *Wound Repair Regen* 18(5):532–542.
24. Munford J, Greenwald L (1974) The hypoglycemic effects of external insulin on fish and frogs. *J Exp Zool* 190(3):341–346.
25. Mena EE, et al. (1990) Conantokin-G: A novel peptide antagonist to the N-methyl-D-aspartic acid (NMDA) receptor. *Neurosci Lett* 118(2):241–244.
26. Craig AG, et al. (1999) Contulakin-G, an O-glycosylated invertebrate neurotensin. *J Biol Chem* 274(20):13752–13759.
27. Cruz LJ, et al. (1987) Invertebrate vasopressin/oxytocin homologs. Characterization of peptides from Conus geographus and Conus straitus venoms. *J Biol Chem* 262(33): 15821–15824.
28. Parkes DG, Mace KF, Trautmann ME (2013) Discovery and development of exenatide: the first antidiabetic agent to leverage the multiple benefits of the incretin hormone, GLP-1. *Exp Opin Drug Discov* 8(2):219–244.
29. Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: Discriminating signal peptides from transmembrane regions. *Nat Methods* 8(10):785–786.

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