

Peptide Neurotoxins from Fish-Hunting Cone Snails

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Animal venoms have received increasing attention from physiologists and biochemists because their potency is often a result of specific blocking of key elements in nerve and muscle cell membranes. Biochemical studies of venoms from two marine snails, *Conus geographus* L. and *Conus magus* L., have uncovered a rich source of such toxins.

worms, while smaller numbers prey on other mollusks or on fish (1). The shells of some piscivorous cones are shown in Fig. 1. Fish-hunting snails have special problems because their prey are much more mobile and can move in three dimensions rather than two. There must therefore be strong selection for venoms that cause very rapid paralysis. A vio-

Summary. To paralyze their more agile prey, the venomous fish-hunting cone snails (*Conus*) have developed a potent biochemical strategy. They produce several classes of toxic peptides (conotoxins) that attack a series of successive physiological targets in the neuromuscular system of the fish. The peptides include presynaptic ω -conotoxins that prevent the voltage-activated entry of calcium into the nerve terminal and release of acetylcholine, postsynaptic α -conotoxins that inhibit the acetylcholine receptor, and muscle sodium channel inhibitors, the μ -conotoxins, which directly abolish muscle action potentials. These distinct peptide toxins share several common features: they are relatively small (13 to 29 amino acids), are highly cross-linked by disulfide bonds, and strongly basic. The fact that they inhibit sequential steps in neuromuscular transmission suggests that their action is synergistic rather than additive. Five new ω -conotoxins that block presynaptic calcium channels are described. They vary in their activity against different vertebrate classes, and also in their actions against different synapses from the same animal. There are susceptible forms of the target molecule in peripheral synapses of fish and amphibians, but those of mice are resistant. However, the mammalian central nervous system is clearly affected, and these toxins are thus of potential significance for investigating the presynaptic calcium channels.

We present in this article an overview of these venoms, and our most recent findings on the presynaptic toxins, including the biological properties and amino acid sequences of five new peptide toxins. One of these has been successfully synthesized. We discuss the strategic advantages to the snails in their producing many small peptides.

The cone snails are a large genus of gastropod mollusks, all of which are believed to be actively venomous predators. Kohn has divided the approximately 300 species of *Conus* into three groups, depending on their major prey; the majority feed on various marine

lently jerking fish, even if unable to escape, could injure the snail and eventually attract the unwelcome attention of other predators. Observation of live snails in aquariums indicates that their sting results in complete immobilization of fish within a few seconds.

Relative to their body sizes, the piscivorous cones have a much larger venom apparatus than do other *Conus* species although the anatomical structures are basically the same (2). The venom is synthesized in a long tubular duct from which it is squeezed by a muscular bulb, and injected into the prey through a hollow radular tooth. These disposable

teeth are like barbed needles and thus are difficult for a prey to dislodge. Typically, a cone snail stores reserve teeth in an organ known as the radular sac.

There are at least two strategies for the snail to come within striking range of a fish. One group of cones including *Conus geographus* and *Conus tulipa* appear to rely on casual contact, scooping small fish in their funnel-shaped rostrum and stinging them. Other piscivorous cones use their brightly colored proboscis to lure prey. These species remain buried under the substrate until they detect a fish (the mechanism of detection is not known). These cones then extend their proboscis above the substrate and wiggle it. A fish so baited may be stung in the mouth and paralyzed almost instantly. The snail then emerges, engulfs the fish with its distensible stomach, and digests it, the scales and bones being regurgitated some hours later (Fig. 2).

We have made a systematic study of the venoms of two fish-eating cones, *C. geographus* and *C. magus* (Fig. 1). The most dangerous of the group is *C. geographus*. A 3- to 5-inch animal can kill a man, and about 20 fatalities have been recorded (3). In contrast, the much smaller *C. magus* appears not to have been responsible for any human deaths. These two species are thought to be only distantly related and have different capture strategies. However, the biochemical strategies used to produce rapid paralysis of fish are the same. Briefly, the venoms contain peptide toxins that attack various critical targets in the neuromuscular system of the prey.

Physiological and Biochemical Complexity of *Conus* Venoms

The first evidence for the existence of multiple toxins in *Conus* venoms came from the physiological studies of Edean and his co-workers (4). These investigators, using the crude venoms from several *Conus* species, found a rather complex pharmacological picture that suggested the presence of more than one active component.

Definitive evidence for several different classes of toxins has been obtained from biochemical studies on fractionation of the venom of *C. geographus* (5-

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Table 1. General properties of conotoxins.

	Venom source		Symptoms in mouse*	Physiological target	No. of amino acids	Net charge pH 7	S-S bridges	Unusual amino acids	References
	<i>C. geographus</i>	<i>C. magus</i>							
α -Conotoxins (GI, GIA, GII, MI)	+	+	Paralysis, death	Acetylcholine receptor	13-15	1½-3½	2		(6,7,11,17-19)
μ -Conotoxins (GVIIIA, GVIIIB, GVIIIC)	(+)	(+)	Paralysis, death	Muscle Na ⁺ channels	22	6-7	3	Hy [†]	(5,9,10,14)
ω -Conotoxins, (GVIA, GVIB, GVIC, GVIIA, GVIIIB, MVIIA)	+	?	"Shaker"	V-sensitive presynaptic Ca ²⁺ channels	25-29	4-7	3	Hy [†]	(12, 15), this work
K-Conotoxins	?	+	?	? K channels	~25	(+)	(3)		(21)
"Sleeper" (GV)	+	?	"Sleep"	Unknown	17	-8	0	Gla	(13)
Conopressin	+	(+)	"Scratching"	? Smooth muscle	9	3	1		(16)
Convulsant (GIV)	+	+	Convulsions, death	Unknown	~100	(-)			(8)

*Intracerebral injection. [†]Hydroxyproline.Table 2. Amino acid sequences of conotoxins. An asterisk indicates that the α -carboxyl is known to be amidated; absence of an asterisk indicates that no assignment has yet been made. The various ω -conotoxins described in this work were reduced and carboxymethylated and analyzed on a Beckman 890D spinning-cup sequencer, as described previously (15). Amounts analyzed were approximately 1 to 5 nmol. Except for γ (γ -carboxyglutamate) and Hy (*trans*-4-hydroxyproline), the standard one-letter abbreviations for amino acid residues are used: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; and Y, tyrosine.

										Reference																							
α	GI	E	C	C	N	P	A	C	G	R	H	Y	S	C*	6,7																		
	GIA	E	C	C	N	P	A	C	G	R	H	Y	S	C	G	K*	6,7																
	GI	E	C	C	H	P	A	C	G	K	H	F	S	C*		7																	
	MI	G	R	C	C	H	P	A	C	G	K	N	Y	S	C*		17,18																
μ	GVIIIA	R	D	C	C	T	Hy	Hy	K	K	C	K	D	R	Q	C	K	Hy	Q	R	C	C	A*	9,10,14									
	GVIIIB	R	D	C	C	T	Hy	Hy	R	K	C	K	D	R	R	R	C	K	Hy	M	K	C	C	A*	10,14								
	GVIIIC	R	D	C	C	T	Hy	Hy	K	K	C	K	D	R	R	R	C	K	Hy	L	K	C	C	A	14								
ω - "Shaker- Blocker"	GVIA	C	K	S	Hy	G	S	S	C	S	Hy	T	S	Y	N	C	C	R	-	S	C	N	Hy	Y	T	K	R	C	-	-	Y*	15	
	GVIB	C	K	S	Hy	G	S	S	C	S	Hy	T	S	Y	N	C	C	R	-	S	C	N	Hy	Y	T	K	R	C	-	-	Y	G	This work
	GVIC	C	K	S	Hy	G	S	S	C	S	Hy	T	S	Y	N	C	C	R	-	S	C	N	Hy	Y	T	K	R	C				This work	
ω - "Shaker"	GVIIA	C	K	S	Hy	G	T	Hy	C	S	R	G	M	R	D	C	C	T	-	S	C	L	L	Y	S	N	K	C	R	R	Y	This work	
	GVIIIB	C	K	S	Hy	G	T	Hy	C	S	R	G	M	R	D	C	C	T	-	S	C	L	S	Y	S	N	K	C	R	R	Y	This work	
	MVIIA	C	K	G	K	G	A	K	C	S	R	L	M	Y	D	C	C	T	G	S	C	R	-	-	S	G	K	C*			This work		
"Sleeper"	GV	G	E	Y	Y	L	Q	Y	N	Q	Y	L	I	R	Y	K	S	N*													13		

Table 3. Activity of ω -conotoxins in vertebrates; i.p., intraperitoneal; i.c., intracerebral.

Toxin	Mouse i.p.	Mouse i.c.	Fish i.p.	Frog neuromuscular junction	References
ω -Conotoxin	GVIA	No effect	"Shaker"	Paralysis, death	Blocks presynaptic Ca channels (12,15)
	GVIB	No effect	"Shaker"	Paralysis, death	Blocks presynaptic Ca channels This work
	GVIC	No effect	"Shaker"	Paralysis, death	Blocks presynaptic Ca channels This work
ω -Conotoxin	GVIIA	No effect	"Shaker"	Paralysis, death	No effect This work
	GVIIIB	No effect	"Shaker"	Paralysis, death	No effect This work
ω -Conotoxin	MVIIA	No effect	"Shaker"	Paralysis, death	No effect This work
α -Conotoxins	Paralysis, death	Paralysis, death	Paralysis, death	Blocks acetylcholine receptor	(6,7,17)
μ -Conotoxins	Paralysis, death	Paralysis, death	Paralysis, death	Blocks muscle Na channels	(5,10,14)

16) (Table 1). We have used three assays for detecting active components and following their purification: intraperitoneal injection into mice or into fish and intracerebral injection into mice. Surprisingly, all peptides that were active intraperitoneally in mice were also active intracerebrally, but it is not completely clear whether some of this might be due to escape of peptide into the periphery. The reverse is not true, several of the intracerebrally active peptides being inactive in the mouse intraperitoneal assay. Because of the conspicuous lethal activity of some toxins, the effects of others are masked in the crude venom and are only apparent in relatively pure fractions. One unexpected finding from this work is the degree to which different vertebrate groups vary in their response to some of the toxins. In particular, fish are paralyzed by more of the toxins than are mice.

With the exception of the convulsant, which is a small protein (8), all the toxins isolated so far are relatively small peptides (Tables 1 and 2). Most are strongly basic molecules with from one to three disulfide bridges. All that have been adequately studied have been shown to be amidated at the carboxyl terminus.

Nonparalytic Toxins

The venoms contain a group of toxins able to paralyze directly the neuromuscular system of fish. However, the last three classes in Table 1 (the sleeper peptide, the convulsant toxin, and conopressin) do not appear to have any direct effect on fish when injected intraperitoneally.

The effects of the sleeper peptide (13) in mice are unusual in that intracerebral injection produces a prolonged sleeplike state from which the mice recover in from 12 to 24 hours. Its chemistry is different from that of other conotoxins. Although amidated at the carboxyl end, the sleeper peptide is extremely acidic because of the presence of five residues of γ -carboxyglutamate, and it contains no cystine. The modified residues are necessary for sleeper activity but not sufficient: the peptide is inactivated by decarboxylation of the γ -carboxyglutamate, as well as by removal of the two carboxyl terminal amino acids leaving all the γ -carboxyglutamate residues intact.

The convulsant mentioned above, a small protein of approximately 100 amino acids, is also without apparent effect on fish, as is the recently isolated vasopressin homolog (conopressin) (16). When mice are injected intracerebrally

with the conopressin, they scratch themselves repeatedly, an effect which is also produced by injection of vasopressin. Chemically, the convulsant is acidic, whereas conopressin is basic.

Paralytic Toxins: Postsynaptic

The first *Conus* toxins for which chemical structures were determined were the α -conotoxins. The mouse intraperitoneal assay was used to follow the purification of three homologous peptides from *C. geographus* (7) and one from *C. magus* (17). The in vivo effect of the purified toxins was similar to that of whole venom; electrophysiological analysis showed that the peptides acted at the acetylcholine receptor of the neuromuscular junction. These toxins are small (13 to 15 amino acids) and have been synthesized chemically (11, 18, 19). By analogy with the snake toxins that act at the same site, we designated this series of peptides as the α -conotoxins.

The pharmacological results of Ender and co-workers (4) indicated that there was likely to be a second class of toxins that specifically blocked sodium channels in muscle. The muscle sodium channel toxins have been isolated (5, 9, 10, 14), and three homologous peptides have been purified and sequenced. They are larger than the α -conotoxins, with 22 amino acids and three disulfide bridges and contain 3 moles of hydroxyproline in sequences that are unrelated to those in which this modified amino acid occurs in collagen. These toxins show high specificity for muscle sodium channels, with essentially no effect on those of nerve. We refer to these peptides as μ -conotoxins. No μ -conotoxin has yet been isolated from *C. magus* venom, but the work of Ender and colleagues (4) on crude venom suggests that such a peptide may be present. We have preliminary evidence for hydroxyproline-containing peptides in a fraction that is highly active in the mouse intraperitoneal assay.

Paralytic Toxins: Presynaptic

The *Conus* venoms also contain the ω -conotoxins, or "shaker" peptides. These were so named because they elicit in the mouse intracerebral assay a persistent tremor, which appears a few minutes after injection. Time of onset and duration of the symptoms are dose-dependent. At high concentrations of the toxin (>2 nmol per mouse), the tremors may persist for 5 days, with the mouse being able to carry on many of its normal

activities. The major peptide responsible for the shaker activity was purified from *C. geographus* venom (15). This toxin (ω -conotoxin GVIA), when tested on a nerve-muscle preparation from frog, produced an irreversible block of the presynaptic terminus. Closer analysis showed that the peptide acts by blocking voltage-activated calcium channels, thereby preventing release of acetylcholine (12).

Additional peptides with shaker activity have been detected and purified from both *C. geographus* and *C. magus* venoms (Fig. 3). Amino acid sequences of the purified peptides are given in Table 2. The physiological activities were examined in the frog neuromuscular preparation; the results as shown (Table 3) demonstrate that the venom of *C. geographus* contains two classes of shaker peptides: one blocks the amphibian synapse, and one does not. The single shaker peptide isolated from *C. magus* venom is in the nonblocking category. Although all ω -conotoxins are structurally homologous, there are clearly two subsets corresponding to the different physiological activities. In our system we designate them as ω -conotoxins Type VI and VII, but we informally call them ω -blockers and nonblockers in referring to their effects on frog neuromuscular junction.

One of the ω -conotoxins has been synthesized by solid-phase methods (20). Because of the absence of hydroxyproline, the *C. magus* peptide was the most straightforward target. The synthetic material had all the biological effects of the natural toxin, including shaker symptoms in mouse, fish paralysis, and a lack of activity toward the frog neuromuscular junction. In addition, the synthetic peptide was identical to the authentic natural peptide by various chromatographic tests. The synthesis of an active ω -conotoxin demonstrates that the biological effects observed are due to the naturally isolated peptides and not to minor contaminants.

Recognizing that the differences between "blockers" and "nonblockers" might reflect variation among the target molecules, we examined the behavior of the various shaker peptides toward different vertebrate species (Table 3). All the peptides produced the characteristic symptoms on intracerebral injection into mice, but none produced any visible effect when injected intraperitoneally; however, all produced paralysis and death after intraperitoneal injection into fish, the natural prey of the snails. The two groups differ in their reactions toward frog synapse.

The most recently discovered toxin is a peptide from *C. magus*. It is similar in size to the ω -conotoxins, but seems to affect both pre- and postsynaptic potassium channels (21). This peptide is not yet available in adequate amounts for a full characterization.

Amino Acid Sequences: Polymorphism

Sequences of all known conotoxins are given in Table 2. Two types of variation are found within the classes of toxins in *C. geographus*, in addition to the interspecific changes.

1) There is polymorphism at the level of nucleotide coding for protein, as exemplified by GI compared to GII, or GIIIA compared to GIIIB compared to GIIIC. All individual changes of this type can be ascribed to single-base nucleotide substitutions. Some of the polymorphism is clearly due to variation among members of the snail population. Most of our work has been done with venom pooled from many snails; nevertheless, there is marked variation from batch to batch in the proportions of the α -conotoxins GI and GII. In our first preparation the ratio of GI to GII was greater than 9:1, whereas in the second it was close to 1:1. A few experiments on venoms from individual snails show that some have both forms of the toxin.

2) Second, there is variability at the carboxyl ends of some peptides, which suggests different levels of processing of a precursor. In all the predominant forms of toxins the alpha carboxyl is amidated. This modification typically is carried out by splitting a longer peptide at a pair of basic amino acids, degrading with a carboxypeptidase until a Gly residue is reached, and then removing the carbon skeleton of the glycine, leaving its amino group as the attached amide (22). Such a process could explain the presence of GIA, and of GVIB, while GVIC could arise by continued action of the carboxypeptidase. Whether the minor variants are produced in this way can only be decided by direct biochemical analysis, or by examining the nucleotide sequences of the corresponding genes.

Venoms as Weapons

The biological success of the cone snails depends on their highly developed venoms and delivery systems. *Conus* species are among the dominant predators in tropical marine communities. Those cones that depend on killing fish have taken two biochemical strategies to



Fig. 1. Shells of several *Conus* species. (Center) The geography cone, *Conus geographus* Linné (Marinduque Island, Philippines). From top clockwise: the magus cone, *Conus magus* Linné (Sulu Sea); the bubble cone, *Conus bullatus* Linné (Samar Island, Philippines); *Conus kinoshitai* Kuroda (Bohol Island, Philippines); *Conus gubernator* Hwass (Madagascar); *Conus chusaki* da Motta (Phuket, Thailand); *Conus barthelemyi* Bernardi (Mauritius); *Conus dusaveli* H. Adams (Balut Island, Philippines). The two species that are the subject of this article, *C. geographus* and *C. magus*, have been observed to hunt fish; most of the other species are likely to be piscivorous, as judged by their shell morphology. [Photo by K. Matz]



Fig. 2. Feeding sequence of the piscivorous eastern Pacific species, *Conus purpurascens*. Top left, the buried *Conus* extends its reddish proboscis above the sand when it detects the presence of a fish. Top right, the fish attempts several times to bite the wormlike proboscis; the snail draws in the proboscis until it has located the fish, which it then stings in the mouth. Middle and bottom, the paralyzed fish lies helpless, and the snail emerges to engulf it with its distensible stomach. A few hours later, only the bones and scales of the fish will be regurgitated by the snail. [Photo by A. Kerstitch]

their limits. First, their venoms contain much smaller peptides than is typical of most groups. Second, they produce an array of toxins that are targeted at different sites in the fish's neuromuscular system.

Five of the eight known classes of conotoxins directly paralyze fish when injected intraperitoneally. They are relatively small peptides (13 to 29 amino acids) compared with the neurotoxins of other venomous animals such as snakes, scorpions, spiders, sea anemones, and heteronemertines, whose toxins consist of 40 to 80 amino acids (23). Only the neurotoxins of hymenoptera venoms are comparable in size, although they are two to three orders of magnitude less potent (24). As with many secreted peptides and proteins the conotoxins are cross-linked by disulfide bridges, 25 to 30 percent of the residues being taken up in this way.

A likely advantage to the cone is that the small size of the peptide enhances permeability, especially through tissues. We have some evidence that the conotoxins reach their target more rapidly than do the larger snake toxins. Our mouse intraperitoneal assay measures the time after injection until death of the animal and is linear with respect to the reciprocal of the dose (6). Under high dose conditions the time can be reduced only to a certain point, which we interpret as being linked to "delivery time," including diffusion. This is much less for the α -conotoxins (2 to 4 minutes) than it is for α -bungarotoxin (10 minutes), despite the much higher affinity of α -bungarotoxin for the acetylcholine receptor. A second advantage could be in the higher molar concentrations achievable with smaller peptides, when the total concentration of material in the venom becomes limiting.

Offsetting these potential advantages of small peptides is their less well-defined structure in solution. Typically, many conformations are available to such molecules, which lack the stabilizing influences of a large number of weak interactions. Since only a small subset of conformations will be complementary to the binding site on the target, the binding energy is effectively reduced by the unfavorable conformational equilibrium. The problem may well be exacerbated by the very high positive charge density (Table 1) of the neurotoxins, tending to stretch out the molecules. Disulfide bridges have long been recognized as providing stability in such situations, and an inverse correlation exists between the size of peptide and the proportion of its resi-

dues taken up in this form. This is especially true of peptides that are secreted into an external medium. The rigidity of the α -conotoxin MI (2 disulfides in 14 residues) is shown by our separation of conformational isomers by high-performance liquid chromatography (18).

The five different kinds of paralytic toxins synthesized by the cone may appear to be "overkill." Most, if not all, are present in the venoms of two distantly related species, a finding that implies that there may be advantages to this strategy. Bee and wasp venoms also contain multiple toxins, as do those of several snakes and scorpions, but the multiplicity is less extreme. Possibly the snail may need to paralyze its prey immediately, as the fish is much more agile and can move in three dimensions rather

than two. Prey are stung at a single point—for example, the mouth—but the target tissues are elsewhere, especially the body musculature near the tail. By the time the venom reaches the target, it is diluted. Under these conditions it is much more effective to produce a unit concentration of each of several consecutive toxins than to produce a several-fold higher concentration of one toxin. If the effects should be multiplicative rather than additive, then a given level of paralysis could be achieved with a much lower total dose of peptides. Preliminary experiments with mixtures of purified paralytic toxins are consistent with this hypothesis; quantitative experiments are in progress.

Nonparalytic Peptides and Relation to the Peptide Neurotoxins

In addition to the direct paralytic toxins there are others that have no overt action when injected intraperitoneally into fish, although they produce distinct symptoms when injected intracerebrally into mice. Two of these, the "sleeper" and "scratcher" peptides, can be considered as having plausible accessory roles in immobilizing prey.

The sleep-inducing peptide is extraordinarily negatively charged, having 5 moles of Glu. This amino acid, which occurs in prothrombin, other blood-clotting enzymes, and a few other proteins, is usually related to calcium binding (25). In the mouse brain, calcium concentrations are too high for the toxin to act simply by depleting the extracellular pools. More likely it acts on some critical site when bound as a calcium complex. Neuromuscular synapses of fish may have such sites, but injecting pure peptide could be ineffective because of non-specific adsorption en route. However, complexed ionically with other basic conotoxins the sleep-inducing peptide may be able to reach adequate concentrations in the synaptic cleft, where it would be released as the positively charged toxins bind to their targets.

The presence of a vasopressin-like peptide in the cone venom is surprising (16). No such molecule had been isolated from an invertebrate, although immunofluorescence staining had suggested the presence of neurophysin- and vasopressin-like materials in insect nervous systems (26). The intense rapid pressor activity of high doses of the hormone in mammals stems from its effects on arterial smooth muscle, with different vascular beds being affected differently. A similar

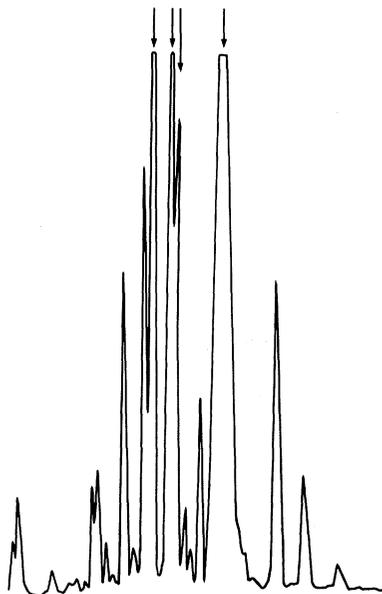


Fig. 3. Multiple shaker peptides in *Conus geographus* venom. One gram of crude lyophilized venom was extracted (15) and the soluble protein (approximately 200 mg) was fractionated on a Sephadex G-25 (column 2.5 by 160 cm). Various biologically active peptides were detected in the eluate, after intracerebral injection into mice. The fractions containing shaker activity were pooled and lyophilized; the peptides were then redissolved and purified by high-performance liquid chromatography as described previously (11: Altex model 2A system, 214 nm; Vydac C18 column, 1 by 25 cm; linear gradients of acetonitrile in 0.1 percent trifluoroacetic acid). The figure shows the elution pattern obtained in a typical run, with arrows indicating GVIC, GVIA, GVIB, and GVIIA in that order (see Table 2). Material from these peaks was repurified with 0.05 percent *n*-heptafluorobutyric acid acetonitrile. Conotoxin GVIB was obtained in a different batch of venom: it is a minor peptide that elutes just after GVIA. Relative amounts of the toxins vary in different preparations, and in different fractions from Sephadex G-25. Conotoxin MVIIA was purified from venom of *C. magus* by similar methods.

effect in fish might well result in preferential shunting of blood to the skeletal muscles, and more effective delivery of the directly paralytic toxins to their targets.

Evolutionary Divergence of Toxins and Targets

The α - and μ -conotoxins block synaptic transmission and muscle action potentials respectively in all vertebrates tested. It seems likely therefore that their target molecules (acetylcholine receptors and muscle sodium channels) are rather stable over evolutionary time. In contrast, the ω -conotoxins vary in their actions according to the test animal. Two types of variability must be distinguished because they have different implications.

The first concerns the actions of a single toxin in different species. ω -Conotoxin GVIA paralyzes fish but not mice after intraperitoneal injection, and electrophysiological tests show that it affects presynaptic calcium channels in the neuromuscular junction of frogs but not mice. Therefore, we can conclude that those channels have undergone significant change during the evolution of the vertebrates.

The second type of variability is between the two series of ω -peptides (GVI and GVII). They are clearly distinct in their actions on calcium channels at the frog neuromuscular junction, one set blocking while the other does not. However, both paralyze fish after intraperitoneal injection, both are inactive intraperitoneally in mice, but both are intracerebrally active. Since these peptides are rather small, we expect them to be monovalent and not to have two independent binding sites. One possible explanation for the physiological differences is that there are at least two types of calcium channels, and that the GVI and GVII series are preferentially target-

ed to one or the other. This is an attractive notion since both series are found in a single venom, and the sequence difference (>50 percent) is such as to suggest a long period of divergence, possibly even longer than that since speciation (27). Since the snail feeds only on fish, and both classes of shakers produce paralysis, it could well be that their primary targets are different. Variants of the calcium channel perhaps occupy distinct sites in the neuromuscular system. For instance, one class of ω -toxins might be targeted to the neuromuscular junction, while the other could preferentially inhibit an earlier synapse in the transmission route. An alternative explanation is that presynaptic Ca^{2+} channels have diverged significantly in different fish prey and the two series of ω -toxins are "tracking" different prey types. Finally, the shaker effects after intracerebral injection of mice are elicited by both sets of toxins, suggesting that a primitive and highly conserved form of calcium channel may be present in the mammalian central nervous system.

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27. In aligning the sequences of ω -conotoxins, we obtain the following minimum mutational changes (ratio of amino acid differences to sites compared; number of insertions or deletions): GVIA versus GVIIA (13/27; 1 gap); GVIA versus MVIIA (12/24; 3 gaps); GVIIA versus MVIIA (8/24; 3 gaps). Depending on how heavily the gaps are weighted, this can be used to argue for or against the idea that divergence of the two classes preceded divergence of the two species. Isolation of a GVI type of "blocking shaker" from *C. magus* would be a clear indication that both classes of toxin have been maintained in both snails for a very long time period. This would tend to support the view that they have physiologically distinct targets in the prey.
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