Conus Venoms: A Rich Source of Novel
Ion Channel-Targeted Peptides

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Terlau, Heinrich, and Baldomero M. Olivera. *Conus* Venoms: A Rich Source of Novel Ion Channel-Targeted Peptides. *Physiol Rev* 84: 41–68, 2004; 10.1152/physrev.00020.2003.—The cone snails (genus *Conus*) are venomous marine molluscs that use small, structured peptide toxins (conotoxins) for prey capture, defense, and competitor deterrence. Each of the 500 *Conus* can express ~100 different conotoxins, with little overlap between species. An overwhelming majority of these peptides are probably targeted selectively to a specific ion channel. Because conotoxins discriminate between closely related subtypes of ion channels, they are widely used as pharmacological agents in ion channel research, and several have direct diagnostic and therapeutic potential. Large conotoxin families can comprise hundreds or thousands of different peptides; most families have a corresponding ion channel family target (i.e., ω-conotoxins and Ca channels, α-conotoxins and nicotinic receptors). Different conotoxin families may have different ligand binding sites on the same ion channel target (i.e., μ-conotoxins and δ-conotoxins to sites 1 and 6 of Na channels, respectively). The individual peptides in a conotoxin family are typically each selectively targeted to a diverse set of different molecular isoforms within the same ion channel family. This review focuses on the targeting specificity of conotoxins and their differential binding to different states of an ion channel.

I. OVERVIEW OF CONE SNAILS AND CONOTOXINS

A. Introduction

The first *Conus* venom peptides were isolated and characterized two decades ago (32, 143), and the systematic investigation of cone snail toxins has continued at an

accelerating pace. These studies have revealed that each of the ~500 different species of predatory cone snails (genus *Conus*) (156) has its own distinctive, complex, and peptide-rich venom. Venom is the primary weapon used by these carnivorous molluscs to capture prey and is also believed to be used defensively and competitively, and possibly for other biological purposes as well (137). Because different cone snail species have divergent biotic

interactions, a corresponding divergence in *Conus* venoms is the expected consequence. Cone snails thrive in tropical marine habitats; the complete web of biological interactions in such marine communities provides a general rationale for why each *Conus* species has evolved its own large molecular repertoire of venom components, different from that of every other *Conus* species (139).

The detailed interactions between an individual *Conus* species and other animals in its environment are mostly unknown; thus the pharmacological spectrum to be found in any venom cannot be predicted a priori. There are probably >100 different venom components per species, leading to an estimate of >50,000 different pharmacologically active components present in venoms of all living cone snails. *Conus* venom components have become known as conotoxins or conopeptides, terms used interchangeably in this review. In the literature, disulfide-rich peptides are referred to as conotoxins; conopeptide is used as a more general term for all peptides found in *Conus* venoms.

This review focuses on the neurophysiology of *Conus* venom components. Since only a miniscule fraction of the total conopeptide diversity has been characterized in detail, a few broad themes are emphasized that should be relevant even for venom peptides yet to be characterized. The molecular targets of individual *Conus* venom components are functionally diverse, including G protein-coupled receptors and neurotransmitter transporters; some *Conus* venom components even have enzymatic activity. However, the majority of biologically active *Conus* venom constituents that are characterized at present are small, structured peptides that target ion channels, either of the ligand-gated or voltage-gated class. It is highly likely that most *Conus* peptides have a specific ion channel as the physiologically relevant target. Thus the work that will primarily be reviewed is the effects of *Conus* peptides on ion channels.

In the last 50 years, the dominant intellectual influence of the Hodgkin-Huxley formulation (70, 71) which led to "the Na channel" and "the K channel" as conceptualized components for action potential generation has given way to a rather messier molecular reality. The K channel is really >80 different genes that can assort in a combinatorial fashion to generate an amazing diversity of tetrameric K channel isoforms (67, 79). A similar situation exists for other ion channel types.

The remarkable molecular complexity of ion channels has been a rich evolutionary substrate for generating effective offensive, defensive, and competitive weaponry for *Conus*. Because these predatory snails are not notable for either speed or mechanical weaponry, to compensate they have evolved rapidly acting, potent venoms. Targeting ion channels is a sensible venom strategy in this biological context: the rich molecular diversity of potential ion channel targets is one factor that leads to molecular complexity of peptides in cone snail venoms.

Two features of individual conopeptides make them of particular interest to the community of neurophysiologists. The first is their ability to discriminate between closely related molecular isoforms of members of a particular ion channel family. Their unprecedented selectivity makes conopeptides an increasingly important tool for defining ion channel function. Most ion channel families have a bewildering array of molecular forms, with the true extent of molecular complexity being undefined in many cases. Pharmacological agents that discriminate between members of a large ion channel family will become increasingly essential for differentiating the physiological roles of closely related ion channel isoforms. A second emerging aspect of conotoxins is their potential utility for investigating different states of the targeted ion channel and transitions between these states. Although such studies are in their infancy, the experimental evidence demonstrating that conotoxin interactions are state dependent and may affect transitions between states is compelling, and will be reviewed below.

Two major sections of this review focus on ligand-gated and voltage-gated ion channels. A theme in the ligand-gated ion channel section is the subtype selectivity of *Conus* peptides for their targets. The section on the interaction of *Conus* peptides with voltage-gated ion channels reviews data that illustrate the state dependence of conotoxin interactions.

B. Cone Snails: Historical Perspectives and Biology

1. Conus *in human history*

The present-day research on cone snails and cone snail venoms is a culmination of the long documented history of human interest of this group of molluscs. The most familiar molluscs are those that are eaten (e.g., oysters, escargots); although cone snails are harvested for food in some Pacific islands, they are not abundant enough to be a notable culinary resource. However, the strikingly beautiful patterns on their shells have attracted human interest from the earliest times, and in a wide variety of cultures. A particularly striking example that may be more than 5,000 years old (shown in Fig. 1) is a *Conus* necklace excavated from a Mesopotamian tomb in Uruk, the city presently believed to be the first urban settlement. Cone snails were known to Roman scholars and natural history collectors, but it was not until the age of European colonization that they attracted wide attention. European collectors were so intrigued by cone snails that several of the rare and striking species shown in Figure 1 became among the most valuable of all natural history objects. The matchless cone, *Conus cedonulli*,

FIG. 1. Shells of cone snails and their use in early human cultures. *A*: an ~5,000-year-old necklace excavated from a tomb in Uruk in the Tigris-Euphrates valley, believed by some archeologists to be the first true city on earth. The necklace, now at the Pergamon Museum, Berlin [Copyright Staatliches Museum zu Berlin, Vorderasiatisches Museum (Inv.-Nr.: VA11091)], has at least two species of cone snails. The larger species, shown in the central section of the necklace is *Conus ebraeus*, the Hebrew cone, and the smaller species is *Conus parvatus*. Freshly collected shells of the two species are also shown in *B* (*top, C. ebraeus*; *bottom, C. parvatus*). Although *Conus parvatus* is found in the Persian Gulf and the Northern Red Sea, the closest collection locality for *C. ebraeus* would be Oman and Masirah Island, at the southwestern tip of the Arabian peninsula; the specimens were presumably traded from there to Mesopotamia. Another possibility is that the specimens were collected off the northwestern Indian subcontinent where *C. ebraeus* is common. There is evidence for trade between Indian Valley civilizations (Harappa and Mohenjo-daro) and Mesopotamia from the earliest times. *C*: seven *Conus* species are shown. The large specimen at the extreme *left* is *Conus marmoreus*, the marble cone, the type species of the genus *Conus*, and at the extreme *right* is the geography cone, *Conus geographus*, the species causing the majority of human fatalities. The large specimen on top is the glory-of-the-sea cone, *Conus gloriamaris*, one of the most prized and valuable natural history objects of the 18th and 19th century. The four smaller shells on the bottom row are, from *left* to *right*, *Conus cedonulli*, the matchless cone, a specimen of which once outsold a masterpiece by Vermeer; *Conus imperialis*, the imperial cone; *Conus purpurascens*, the purple cone; and *Conus magus*, the magician's cone. *C. cedonulli* and *C. imperialis* are vermivorous (worm-hunting) species; *C. gloriamaris* and *C. marmoreus* are snail-hunting species; and *C. purpurascens*, *C. magus*, and *C. geographus* are piscivores (fish-hunting cone snails).

even outsold a painting by Vermeer at auction in the late 18th century (148).

The other aspect of cone snails that has attracted human interest is that they can be deadly to humans (35). One species, *Conus geographus*, has caused multiple human fatalities. Small Pacific island communities were clearly aware of this potential, and two reports from the 19th century (26, 112) show that in some island cultures, an appropriate medical response to a cone snail sting was practiced. The first scientific record of a fatality from a cone snail sting is in the monumental work of Rumphius (157). A few dozen stinging cases by *C. geographus* have

been detailed in the medical literature, and the high frequency of fatality from untreated stings (~70%) of this species has been noted (194). The recognition that this snail could kill people led to the initial scientific investigation of cone snail venoms (47, 98). Earlier scientific work on cone snails that was not purely taxonomic focused on the pharmacological and physiological properties of whole venom. Since these studies were recently reviewed (139), the present article reviews work involving individual venom components.

The increasing interest in cone snail venom components arises in part from their pharmacological potential. Not only are conopeptides used as basic science tools for neurobiologists, but several cone snail toxins are being directly developed for therapeutic applications (86, 127, 138). At present, at least three conopeptides have reached human clinical trials as drug candidates, and several others are being actively explored. The scientific basis for the therapeutic potential of conopeptides is described in the sections that follow.

2. Natural history of Conus: overview of Conus evolution

All cone snails are conventionally included in a single large genus (Conus); however, all venomous snails presently known, including Conus, are usually included in a larger taxon, the superfamily Conacea (also known as the suborder Toxoglossa), collectively referred to as the toxoglossate gastropods (an overview of the group is provided by Taylor et al., Ref. 178). In shallow-water tropical marine environments, cone snails are the most numerous and diverse toxoglossates. However, viewed from a different perspective, Conus are only a minor component of venomous molluscan diversity, probably 10 times as many toxoglossate gastropod species exist outside the genus Conus as within it.

The fossil record of the toxoglossate gastropods suggests origins in the Cretaceous period, but true Conus are known only after the Cretaceous extinction (96). The first fossils that unquestionably belong to the genus Conus are found in the early Eocene. Thus it is generally considered that like many other carnivorous snails, this is a group that had an ecological opportunity with the massive marine extinction at the K-T (Cretaceous/Tertiary) boundary. Predatory Mesozoic marine molluscs, particularly the ammonites, disappeared at the same time as the dinosaurs on land. This major extinction probably made the first radiation of cone snails possible, and a second radiation, at the beginning of the Miocene, basically continues to the present day. It is likely that there are as many living species of cone snails as there ever have been, since the genus appears to have continually expanded. These radiations of Conus in particular, and toxoglossate molluscs

in general, presumably require the evolution of novel venoms and efficient delivery systems for the venoms.

3. Biology of cone snails

The genus Conus is a large and successful group of 500 species (156) of carnivorous predators found in all tropical marine habitats. All members of this species-rich group use venom as the major weapon for prey capture and have a delivery system consisting of a venom duct, where the venom is synthesized and stored; a venom bulb, believed to transfer venom from the duct; and most remarkably, hollow, harpoonlike teeth which serve as a hypodermic needle for injecting the venom. Most cone snails have a long distensible proboscis, and when they forage for prey, a single harpoon tooth is transferred into the lumen of the proboscis. Once the extended proboscis touches prey, the tooth is propelled out, grasped by circular muscles at the anterior tip of the proboscis, and the venom injected into the prey through the hollow tooth. This efficient venom delivery system is characteristic of all cone snails (98), as well as certain other toxoglossate molluscs.

Cone snails are generally divided into three groups, depending on the prey envenomated. The largest class are the vermivorous (worm-hunting) species; most of these feed on polychaetes (a class of segmented marine worms in the phylum Annelida), but a number of species will also attack hemichordate and echiuroid worms. A second major group are the molluscivorous (snail-hunting) species that hunt other gastropods. The final, most remarkable group are the piscivorous (fish-hunting) cone snails; these have venoms that very rapidly immobilize fish (95, 137, 143, 180).

Most cone snails are nocturnal. Although they have two eye stalks, their vision is poor, and their considerable chemosensory prowess is used to track prey. Even though a few species have been adapted to cooler waters, and some Conus species, including some of the largest forms are collected only at depths >100 m, Conus diversity is greatest in the shallow marine habitats of the tropics. In a single coral reef in the central Indo-Pacific, over 30 different species of Conus can be found (97).

The description of cone snails above does not begin to give the reader a sense of their remarkable biological diversity. For example, while all cone snails harpoon their prey, fish-hunters use a single harpoon to capture a fish, while many molluscivorous species repeatedly inject venom into prey after the first attack and have been observed to use over half a dozen harpoons to capture a single prey snail. Even among fish-hunters, the detailed strategy for prey capture is species specific; some of these more subtle aspects of the biology of Conus, described in a recent review (139), can be used to rationalize the

biochemical and pharmacological diversity of conopeptides (see sect. IC3).

C. Overview of *Conus* Venom Components

1. Biochemistry

An overview diagram of peptidic *Conus* venom components is shown in Figure 2. Two broad divisions of venom components are shown: disulfide-rich conotoxins and peptides that lack multiple disulfide cross-links. In the disulfide-rich conotoxins, Cys residues may be found at an unprecedentedly high frequency; most cysteine residues are separated by 0–6 amino acids from the next Cys residue in the primary sequence. In many conotoxins, multiple pairs of adjacent cysteine residues are found. The arrangement of cysteines in the primary sequence is restricted to only a few patterns; in general, each pattern corresponds to a specific disulfide connectivity. Furthermore, the Cys pattern is diagnostic of the gene superfamily that encodes the peptide (see sect. IC2), and in many cases can be indicative of the pharmacological target of the conopeptide.

Several biochemical features of *Conus* peptides are distinctive. Conopeptides are unusually small; most disulfide-rich conotoxins are 12–30 amino acids. In contrast, the size range of polypeptidic toxins from other venoms is typically 40–80 amino acids. Despite their small size, there is a remarkable interspecific sequence divergence, even between homologous conopeptides from closely related *Conus* species (149, 193). Another striking feature of conopeptides is the presence of an unusually diverse complement of posttranslationally modified amino acids, found at a high frequency in some conopeptide families (27). These include some that are well known and widely distributed [hydroxyproline, *O*-glycosylated Ser or Thr (30)] as well as others that are unusual [6-Br-Trp (82), γ-carboxy-Glu (123), D-amino acids (84), sulfated-Tyr (109)]. However, all conopeptides are translated through the conventional ribosomal mechanism, and mRNAs encoding each peptide can be identified in the venom duct.

2. Molecular genetics

Characterization of venom duct cDNA clones established that *Conus* peptides are initially translated as pre-propeptide precursors. The canonical organization of conopeptide precursors consists of a typical signal sequence at the NH_2-terminal end of the open reading frame (the "pre" region), followed by an intervening "pro" re-
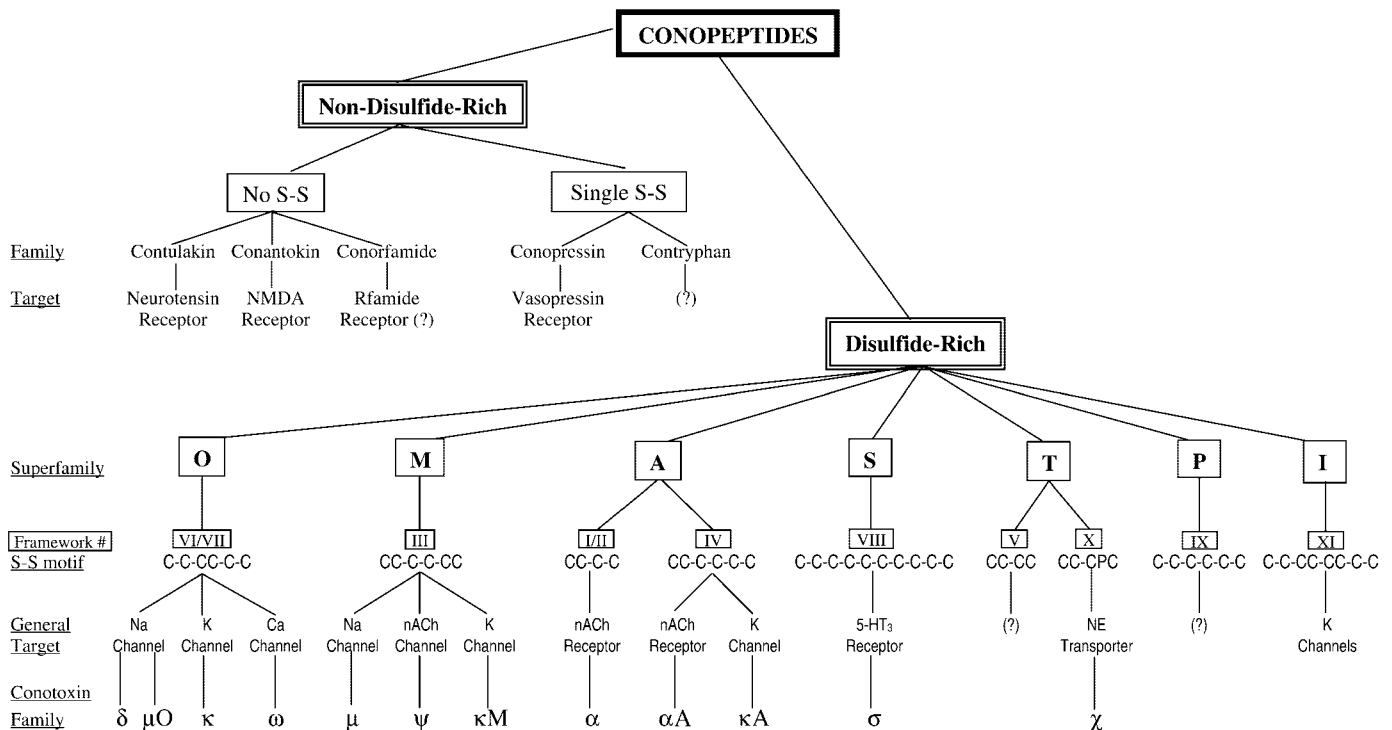

FIG. 2. Organizational diagram for *Conus* peptides, indicating gene superfamilies, disulfide patterns, and known pharmacological targets. *Conus* peptides can be divided into two broad classes: the disulfide-rich conotoxins and nondisulfide-rich peptides. Only the gene superfamilies for the disulfide-rich peptides are shown. The characteristic patterns of cysteine residues in peptides of each superfamily are shown; the nomenclature used is based on that proposed by McIntosh et al. (124) and Olivera (139).

gion, and at the COOH-terminal end of the open reading frame, the mature toxin region, always in single copy. Thus proteolytic cleavage of the precursor to generate the final functional toxin is an obligatory step in the maturation of all *Conus* peptides.

A comparison of homologous cDNA sequences from different *Conus* species revealed an unusual and striking pattern; peptides with similar arrangements of Cys residues in the primary sequence of the mature toxin share a highly conserved signal sequence. These features define members of a conopeptide gene superfamily. A relatively small number of gene superfamilies have undergone extensive proliferation and diversification in the genus *Conus* to generate the majority of the >50,000 different conopeptides found in the venoms of living *Conus* today (137).

Some of the more well-characterized gene superfamilies with the corresponding arrangement of cysteine residues found in superfamily peptides are shown in Figure 2. The mechanisms that lead to the conservation of the signal sequences (a conservation that extends even to the third position of codons, making even silent mutations underrepresented in this region) juxtaposed with the hyperdivergence observed in the mature toxin region (149, 193) remain a subject for speculation; a variety of mechanisms leading to the observed differences in the differential rate of divergence observed for the signal sequence region, the pro region, and the mature toxin region have been proposed (25, 40, 49, 137, 149).

Insights into the mechanism of posttranslational modification of conopeptides have also been obtained, mostly from studies of the best-characterized *Conus* posttranslational modification, the γ-carboxylation of glutamate to the unusual amino acid γ-carboxyglutamate (8, 123). Cone snails express the posttranslational modification enzyme in their venom ducts (9, 174); the enzyme has a recognition signal in the pro region of the precursor. The presence of such a signal in a precursor peptide recruits the posttranslational enzyme and instructs the enzyme to modify specific amino acid residues in the mature toxin region. Thus the pro region of conopeptide precursors provides potential anchor binding sites for posttranslational modification enzymes (72).

3. Overview of pharmacology and physiology

As a group, the cone snails are specialists in neuropharmacology. There are many parallels between conopeptide evolution and modern pharmacological practices. The hypermutation that occurs in conopeptide-encoding regions that accompanies *Conus* speciation is the evolutionary equivalent of a combinatorial library strategy for drug development. Posttranslational modification of conopeptides achieves the same ends as the medicinal chemistry carried out after an initial lead candidate for drug development is identified. A final pharmacological insight regarding the mode of action of conopeptides is that cone snails are sophisticated practitioners of combination drug therapy. To efficiently impose the desired physiological effect on the injected prey, predator, or competitor, multiple conopeptides act together in a synergistic fashion to affect the targeted animal in a manner that benefits the cone snail. The term *toxin cabal* has been applied to an assemblage of conopeptides acting coordinately to a specific physiological end point.

The systematic analysis of the venom components of the fish-hunting species *Conus purpurascens*, the purple cone, defined the presence of two different toxin cabals in the venom (180), whose effects are separable in both time and space. The first, the "lightning-strike cabal," causes immediate immobilization of the injected prey; components of the lightning-strike cabal include peptides that inhibit voltage-gated Na channel inactivation as well as peptides that block K channels. Together, this combination would result in the massive depolarization of any axons in the immediate vicinity of the venom injection site, causing an effect similar to electrocuting the fish. Thus the characteristic tetanic state elicited by the peptides of the lightning-strike cabal are manifest immediately after venom injection.

The second physiological end point is achieved more slowly: a total inhibition of neuromuscular transmission. This is effected by the venom through the "motor cabal" of conopeptides. These peptides act at sites that are remote from the venom injection site, e.g., at neuromuscular junctions. Since transport of the peptides to the remote target sites is required before the desired physiological effects are achieved, the motor cabal's effects are observed after those of the lightning-strike cabal. The motor cabal, found in all fish-hunting *Conus* venoms examined so far, includes peptides that inhibit the presynaptic Ca channels that control neurotransmitter release, the postsynaptic nicotinic receptors, and the Na channels that underlie the muscle action potential.

There is evidence that different fish-hunting *Conus* species may have a divergent spectrum of toxin cabals as part of their repertoire for prey capture. In part, this divergence can be correlated to different behavioral strategies for capturing fish. The combination of lightning-strike and motor cabals is found in the venoms of species that extend their proboscis to initially approach and then harpoon the fish prey. Several species use an alternative strategy for capturing fish; such *Conus* can greatly distend their "false mouths," effectively using them as a net. These *Conus* engulf the fish with the false mouth before they inject venom. Thus the venom of one such species, *C. geographus*, appears to have novel conopeptides that jam the sensory circuitry of the targeted prey; these have been referred to as the "nirvana cabal" (140). It has been proposed that such venom components may be released by the snail to make a school of fish more quiescent, making capture by a net strategy more facile (139).

The combination drug strategy employed by cone snails is presumably one underlying explanation for the complexity of *Conus* venoms, as well as for the surprising pharmacological diversity of *Conus* venom components. Furthermore, different toxin cabals may include peptides that act on the same general class of targets, but through different, and sometimes seemingly incompatible mechanisms. Thus, to interfere with neuromuscular transmission, the motor cabal typically contains a Na channel-targeted peptide that blocks conductance. In contrast, the lightning-strike cabal that causes an immediate immobilization of prey by axonal hyperexcitation requires one or more conopeptides that inhibit Na channel inactivation, thereby increasing the total Na flux with each channel opening event. The former would, on the surface, appear to cancel out the effects of the latter. The evolutionary solution to this problem is of great significance: each peptide is selectively targeted to a specific and different voltage-gated Na channel subtype. As a result, the two peptides are effective components of different cabals, acting within different time windows, without functional cross-interference.

Thus at least two factors favor subtype selectivity of conopeptides. First, cone snails are unusually slow predators lacking effective offensive mechanical weaponry; as a consequence, there is strong selective pressure for the venoms to act very quickly. Binding to an irrelevant target, including those closely related to the physiologically relevant target, would functionally slow down an individual venom component. A second factor is the toxin cabal/combination drug therapy characteristic of the *Conus* strategy; the more complex the venom, the greater the selective pressure for subtype specificity on a newly evolving venom component. The less specific a conopeptide is for its physiologically relevant target, the greater the potential that it would interfere with the activity of another cabal in the venom. The overall complexity of the venom may therefore generally select for individual conopeptides with high targeting specificity.

Thus the biology and evolutionary history of cone snails has led to the unique biochemistry, genetics, pharmacology, and physiology of conopeptides: unusually small, mostly disulfide-rich peptides with a high frequency of nonstandard amino acids, encoded by a few gene superfamilies that have rapidly generated an extraordinarily diverse targeting specificity for ion channels.

II. CONOPEPTIDES TARGETED TO VOLTAGE-GATED ION CHANNELS

A. Introduction and Overview

The voltage-gated ion channel superfamily comprises a large set of structurally similar membrane-bound proteins activated by a change in the transmembrane voltage. These proteins exhibit different selectivity for monovalent cations and are conventionally divided into Na, K, and Ca channels (67). The most important physiological role of voltage-gated ion channels is the generation, shaping, and transduction of the electrical signals of cells. The main pore-forming α-subunit of voltage-gated ion channels consists of either a single subunit containing four homologous domains (Na and Ca channels) or four distinct subunits (K channels); the latter may be either homomeric or heteromeric. The α-subunits interact with auxiliary subunits that are not integral to forming a pore, but which can alter the properties of the α-subunit. Upon activation, voltage-gated ion channels undergo a conformational change, which under physiological conditions results in the selective permeation of cations through the pore of the channel protein. From this open state, voltage-gated ion channels can be either inactivated by an additional conformational change, thereby entering a nonconducting state, or they may be deactivated, returning to a closed state.

Voltage-gated ion channels are targets of toxins from a great variety of different organisms. In this section, we describe the properties of conotoxins interacting with the pore-forming α-subunit of Na, K, and Ca channels; an increasing number of such conotoxin families have been identified (see Fig. 2). Three different *Conus* peptide families are known to target voltage-gated sodium channels: the μ-conotoxins that are channel blockers, the μO-conotoxins that inhibit Na channel conductance, and the δ-conotoxins that delay or inhibit fast inactivation.

One of the ω-conotoxins that block voltage-gated calcium channels, ω-conotoxin GVIA, is probably the most widely used *Conus* peptide in neuroscience; over 2,000 papers in the literature have used ω-GVIA as a pharmacological tool, primarily to inhibit synaptic transmission. *Conus* peptides that target K channels have just begun to be characterized; two peptides, κ-conotoxin PVIIA and κM-conotoxin RIIIK, have been investigated in considerable detail. However, a number of *Conus* peptide families have been shown to also target K channels, but the molecular specificity of most of these has not yet been defined. A striking contrast between peptides that target K channels and those that target Na channels is that the Na channel-targeted *Conus* peptide families appear to be widely conserved over a broad range of *Conus* species. In marked contrast, structurally and genetically divergent *Conus* peptide families have been shown to target K channels in different groups of *Conus* species.

The endogenous functions of peptides that target voltage-gated ion channels are likely to be extremely diverse. In fish-hunting *Conus* venoms, μ-conotoxins targeted to the muscle subtype of sodium channels have a major role in prey paralysis, as do the *Conus* peptides that block the presynaptic calcium channels at the neuromus-

cular junction of the prey. These are therefore major components of the motor cabal of toxins, which cause an irreversible block of neuromuscular transmission. The δ-conotoxins that inhibit Na channel inactivation are a major component of the lightning-strike cabal of conopeptides, as are at least some of the K channel blockers (see sect. ιC3). Thus prey immobilization and paralysis are likely functions of many of these peptides. However, the enormous complexity of Na channel- and K channel-targeted *Conus* peptides suggests that these are likely to have many other endogenous biological functions; for example, some δ-conotoxins could conceivably play a role in predator deterrence, since if targeted to the appropriate Na channel subtype, they would be expected to cause intense pain in an injected animal.

B. Na Channel-Targeted Toxins

Voltage-sensitive Na channels are key molecules for generating action potentials in electrically excitable cells by forming the action potential upstroke. To date, 10 different isoforms of α-subunits with different developmental and tissue distribution have been identified, and a standardized nomenclature ($Na_v1.x$) has recently been proposed (18, 57, 58, 136). Voltage-gated Na channels have conventionally been divided in two pharmacological classes, tetrodotoxin (TTX)-sensitive and TTX-insensitive channels, based on their sensitivity to the classical blocker of Na currents TTX. The interaction site of TTX with the channel protein has been called site I and is postulated to be at the extracellular end of the ion channel pore. In addition to this site, up to five other interaction sites for different substances with different modes of activity on the channel protein can be distinguished (2, 17). Most ligands that target these other sites cause a net increase in Na currents either by shifting the current-voltage relationship or by blocking the fast inactivation of the channels. Na channel-targeted toxins have been important compounds in the historical development of neuroscience and remain indispensable pharmacological tools for neuroscience research.

1. μ-Conotoxins

μ-Conotoxins were originally isolated from the venom of the fish-hunting snail *C. geographus*. These peptides are 22–25 amino acids with 6 cysteine residues arranged in a class III framework (see Fig. 2) and belong to the M-superfamily of conopeptides (see Fig. 2). μ-Conotoxins block Na currents by acting at site I of Na channels, the interaction site of TTX. So far, μ-conotoxins are the only polypeptide toxins known that affect Na currents through this site.

The μ-conotoxins GIIIA and GIIIB from the venom of *C. geographus* are the best characterized. μ-GIIIA is known to specifically block skeletal muscle Na channels ($Na_v1.4$) and exhibits significantly decreased affinity for any other Na channel subtype (10, 34, 60). It is known that μ-GIIIA interacts with the pore region of the ion channel and therefore competes with TTX for binding to site I. Structure-function studies have revealed a rather complex interaction of μ-GIIIA and μ-GIIIB with the ion channel pore, with several amino acids at different positions of the peptide contributing to the high affinity of binding. These data indicate that binding sites for TTX and μ-conotoxins overlap, but are not identical (42, 129); some mutations that have strong effects on the TTX sensitivity of $Na_v1.4$ lead to only a minor increase in the IC_{50} of μ-GIIIA to the channel (19, 21, 175).

From a set of different studies including modeling work, a detailed picture of how μ-conotoxins plug the ion channel pore has been hypothesized (74). The amino acid that likely occludes the pore is Arg-13 of μ-conotoxin GIIIA (10, 56); this residue seems to act as a steric and electrostatic barrier (42, 56, 74). Some mutations at this locus result in analogs of μ-GIIIA/μ-GIIIB that only partially block the channel, presumably because occlusion of the ion channel pore is incomplete. Such analogs are useful as a starting point for evaluating the contributions of other residues within the peptide. Furthermore, μ-GIIIA and μ-GIIIB have been heavily used as tools to investigate structure-function relationships of Na channels. For example, pairwise interactions between the residues of μ-GIIIA and those of $Na_v1.4$ have been investigated by the double mutant cycle method (65, 163). Together with the three-dimensional structure of the peptide, the data clearly indicate that the four repeats within the α-subunit of Na channels have a clockwise orientation (41). Furthermore, a mutant μ-GIIIA (R13Q) has been used to study how the conformation of the pore region near the selectivity filter affects channel dynamics. The data indicate a conformation change in the P-loop of domain IV of Na channels during activation (66).

μ-Conotoxin PIIIA was isolated from the venom of *C. purpurascens*. This peptide also contains an Arg residue (Arg-14), shown to be a key residue for binding, at a position homologous to Arg-13 of μ-GIIIA (168). Therefore, μ-PIIIA shares the critical guanidinium group postulated to be important for interaction with site I of Na channels. Although quite similar to μ-GIIIA, μ-PIIIA has features that make it particularly useful for studying Na channels in situ. In amphibian systems, μ-PIIIA, unlike μ-GIIIA, irreversibly blocks muscle Na channels, providing a useful tool for studying synaptic electrophysiology. When the effects of the peptide on cloned mammalian Na channels are evaluated, the specificity of μ-conotoxin PIIIA is not as highly focused for $Na_v1.4$ as is observed for μ-GIIIA; μ-PIIIA blocks neuronal $Na_v1.2$ Na channels with affinities in the submicromolar range, although $Na_v1.4$ skeletal muscle Na channels are clearly the highest affin-

ity target (168). This feature can be used to subdivide TTX-sensitive Na channels into three categories: *1*) sensitive to μ-GIIIA and to μ-PIIIA (i.e., Na$_v$1.4); *2*) insensitive to μ-GIIIA, but sensitive to μ-PIIIA (Na$_v$1.2 and perhaps others); and *3*) insensitive to μ-GIIIA and μ-PIIIA.

The differential properties of μ-PIIIA have been used for further discrimination of different Na channel subtypes in a number of systems. A recent example is the demonstration that Na$_v$1.2 and Na$_v$1.7 channels appear sequentially during neuronal differentiation of PC12 cell lines (158). Interestingly, in rat brain neurons μ-PIIIA seems to preferentially inhibit persistent over transient voltage-activated Na currents (135).

Recently, the solution structure of μ-PIIIA has been solved by NMR techniques. It was shown that μ-PIIIA in solution adopts two conformations due to a *cis/trans*-isomerization at hydroxyproline-8 (135). The three-dimensional structure of the *trans*-conformation is significantly different from the previously solved structures of μ-GIIIA and μ-GIIIB. These underlying structural differences may be one factor in the divergent pharmacological properties of these peptides.

A new μ-conotoxin that has neuronal subtype specificity for voltage-gated sodium channels was recently reported; this peptide, μ-conotoxin SmIIIA from *Conus stercusmuscarum*, has several distinctive sequence features (see Table 1) (186). Uniquely among Na channel ligands, this peptide inhibited most TTX-resistant Na current irreversibly in voltage-clamped dissociated neurons from frog sympathetic and dorsal root ganglia. The TTX-sensitive Na currents in these neurons were largely unaffected by the peptide. Although the effect of this novel μ-conotoxin on other voltage-gated Na channel subtypes, particularly those neurons from mammalian systems, has not yet been reported, this peptide is clearly different from other μ-conotoxins reported so far; neither μ-conotoxin GIIIA nor PIIIA had an effect on the amphibian TTX-resistant channels. The results suggest that the μ-conotoxin family

is a potential source of subtype-specific voltage-gated Na channel antagonists. Presumably, all μ-conotoxins act on site I; the arginine residue believed to occlude the pore in μ-conotoxins GIIIA and PIIIA is conserved in μ-conotoxin SmIIIA (see Table 1).

2. μO- and δ-Conotoxins

Both the μO- and δ-conotoxins are unusually hydrophobic peptides that belong to the O-superfamily (see Fig. 2), and the pattern of disulfide bonds gives these peptides an inhibitory cysteine knot (ICK) motif.

The μO-conotoxins are a *Conus* peptide family that inhibits Na channel conductance like μ-conotoxins, but most likely through a different mechanism. In contrast to μ-conotoxins, no competition for binding with saxitoxin (STX) has been observed for μO-conotoxins, indicating that these peptides do not interact with site I (181). So far, the mechanism of interaction with Na channels is not understood. Two closely related 31-amino acid peptides, μO-MrVIA and μO-MrVIB (see Table 1) isolated from the venom of the snail hunting species *C. marmoreus*, have a disulfide bonding pattern that more closely resembles the ω-conotoxins (see below) than the μ-conotoxins.

μO-MrVIA blocks Na$_v$1.2 channels expressed in *Xenopus* oocytes in the nanomolar range. Na currents recorded from hippocampal pyramidal neurons in culture are also inhibited by the peptide (181). The blocking effect of μO-MrVIA was readily reversible for the Na currents recorded from hippocampal cells in culture; in contrast, it was only very slowly reversible for Na$_v$1.2 currents recorded in the *Xenopus* expression system. Unlike TTX/STX, μO-MRVIA did not show any phasic or use-dependent inhibition of Na currents, but the steady-state inactivation curve of the Na currents is shifted to more hyperpolarized potentials in the presence of the peptide. μO-MrVIA appears to block both TTX-sensitive

TABLE 1. *Conopeptides targeted to Na channels*

Peptide	*Conus* Species	Subtype Targeted	Sequence	Reference Nos.
μ-Conotoxins				
μ-GIIIA	*C. geographus*	Muscle	RDCCTOOKKCKDRQCKOQRCCA*	33, 161
μ-GIIIB	*C. geographus*	Muscle	RDCCTOORKCKDRRCKOMKCCA*	33, 161
μ-PIIIA	*C. purpurascens*	Diverse TTX sensitive	ZRLCCGFOKSCRSRQCKOHRCC*	168
μ-SmIIIA	*C. stercusmuscarum*	TTX insensitive (amphibian)	ZRCCNGRRGCSSRWCRDHSRCC*	186
μO-Conotoxins				
μO-MrVIA	*C. marmoreus*	(Diverse)	ACRKKWEYCIVPIIGFIYCCPGLICGPFVCV	121
μO-MrVIB	*C. marmoreus*	(Diverse)	ACSKKWEYCIVPILGFVYCCPGLICGPFVCV	121
δ-Conotoxins				
δ-PVIA	*C. purpurascens*	(Diverse)	EACYAOGTFCGIKOGLCCSEFCLPGVCFG	166
δ-SVIE	*C. striatus*	(Not established)	DGCSSGGTFCGIHOGLCCSEFCFLWCITFID	13
δ-TxVIA	*C. textile*	(Molluscan subtype)	WCKQSGEMCNLLDQNCCDGYCIVLVCT	69
δ-GmVIA	*C. gloriamaris*	(Not established)	VKPCRKEGQLCDPIFQNCCRGWNCVLFCV	167

O, *trans*-4-hydroxyproline; TTX, tetrodotoxin. * Amidated COOH terminus.

and TTX-insensitive Na channels, but the complete subtype specificity of the peptide remains to be established.

The main effect of conopeptides belonging to the δ-conotoxin family is the inhibition of the fast inactivation of Na currents, one of the key mechanisms underlying the proper shape and duration of action potentials. This results in a hyperexcited state of the affected cells, which can eventually lead to a massive electrical hyperexcitation of the complete organism. The molecular mechanism of this action is not yet clear, but extracellular binding of the peptides seems to affect events in the intracellular part of the Na channel important for fast inactivation. δ-Conotoxins have the same cysteine framework as the μO- and ω-conotoxins and belong to the same O-conotoxin superfamily (Fig. 2)

The high degree of hydrophobicity that is a biochemical hallmark of both the μO- and δ-conopeptides might be important for the mechanism of action. The most recent structure information on gating of voltage-activated K channels (80, 81) is likely to be highly relevant in providing a rationale for the observed hydrophobicity of these peptides. However, similarly detailed structural information of comparable resolution for voltage-gated sodium channels is unavailable, and the peptide binding sites have not been directly localized; thus any detailed hypothesis would be conjectural. Several δ-conotoxins from fish- and snail-hunting species have been identified (see Table 1).

The observed effects of the δ-conotoxins can depend enormously on the system being investigated. δ-TxVIA from *Conus textile* (originally called the "King Kong" peptide because it causes a characteristic behavior upon injection into lobster) was shown to prolong Na currents in molluscan neuronal membranes. In vertebrate systems, this peptide binds to Na channels but without any toxic effects. δ-PVIA was isolated from the venom of *C. purpurascens*, a fish-hunting *Conus* species; the peptide elicits excitatory symptoms in mice and fish but is inactive in molluscs even at doses 100-fold higher. When administered to fish, it causes specific muscle contraction resulting in a characteristic extension of the mouth, which was termed "the lockjaw syndrome" (166). δ-PVIA slows fast inactivation of $Na_v1.2$-mediated currents expressed in the *Xenopus* expression system, as well as of Na currents recorded from hippocampal neurons in culture (180). More recently, it was demonstrated that the peptide also affects $Na_v1.4$- and $Na_v1.6$-mediated currents (158). In rat brain synaptosomes, δ-PVIA competes with δ-TxVIA for the same binding site, despite the latter being nontoxic in vertebrate systems (166).

δ-PVIA is one of the major constituents of the lightning strike cabal in *C. purpurascens* venom. The excitatory effects of δ-PVIA act synergistically with the K channel-blocking peptide κ-PVIIA, resulting in the almost immediate immobilization of the prey (see sect. IIC1). δ-GmVIA isolated from *Conus gloriamaris* causes action potential broadening in *Aplysia* neurons (64, 167). Furthermore, this peptide has micromolar affinity for $Na_v1.2$ and $Na_v1.4$ but does not affect $Na_v1.6$-mediated Na currents.

C. K Channel-Targeted Toxins: κ-, κA-, and κM-Conotoxins

K channels are not only important for the repolarization phase of action potentials, but are also involved in setting the resting membrane potential, bursting activity, and have a variety of specialized purposes in a wide range of cell types (67). In accordance with this broad spectrum of different physiological functions, more than 80 genes encoding different K channels have been identified, and several families of voltage-gated K channels ($K_V1.x$, $K_V2.x$, etc.) are known. The α-subunits of voltage-gated K channels, the first class to be elucidated, are proteins with six transmembrane domains. The functional pore-forming protein complex may either be homomeric (with 4 identical subunits) or heteromeric (with 2 or more different α-subunits). The first K channel-targeting conotoxin was identified relatively recently (180), and only a few K channel-targeted conopeptides have been extensively characterized. This may have to do with the potential high specificity anticipated for these peptides: if the target of a conopeptide were a heteromeric K channel, it will be a challenge to define the high-affinity K channel target affected by the conopeptide. Recent results indicate that the cone snails have evolved many different families of K channel-targeting peptides, although at present, the pharmacological range and specificity of any family, particularly in mammalian systems, is largely undefined

1. κ-Conotoxins

The first conotoxin shown to target voltage-gated K channels was κ-conotoxin PVIIA, isolated from the fish-hunting cone *C. purpurascens* (169, 180). With the use of the *Xenopus* expression system, it was demonstrated that this peptide blocks *Shaker* K current with relatively high affinity (IC_{50} 50 nM). Because the *Shaker* channel was cloned from *Drosophila*, this is not the natural target of κ-PVIIA; the latter has not been identified. Nevertheless, κ-PVIIA seems to be quite subtype selective; it was shown to differentiate between splice variants of the *Shaker* channel homolog from lobster (91). Injection of κ-PVIIA leads to excitatory symptoms in mice, but to date no cloned mammalian K channel (>20 tested so far) has been shown to be affected by this toxin.

The K channel block of κ-PVIIA is physiologically significant for prey capture as it is a key venom component in the rapid immobilization of the fish prey. The activity of κ-PVIIA acts in a synergistic manner with δ-conotoxin PVIA, leading to massive hyperexcitation of

the injected animal, resulting in an almost instant tetanic paralysis. Therefore, κ-PVIIA plays a very critical role in the lightning-strike cabal of conopeptides.

The interaction of the peptide with the ion channel pore is a bimolecular reaction. The analysis of *Shaker* K channel block by κ-PVIIA revealed that the conopeptide binds to the extracellular mouth of the ion channel pore (see Fig. 3). With the use of alanine-scanning mutagenesis, the interaction surface of κ-PVIIA with the *Shaker* channel pore has been identified. Mutations within the P-loop of the channel protein have major effects on the binding of κ-PVIIA (169); a double mutant cycling analysis revealed that κ-PVIIA, like other K channel blocking peptides, contains a critical dyad motif of amino acids important for K channel blockade (78). This dyad consists of lysine-7 and a phenylalanine-9, which are ~6 Å apart. K7 is believed to occlude the ion permeation pathway, and the hydrophobic amino acid F9 may be important for hydrophobic interactions of the peptide with the ion channel.

These data support the critical dyad model developed by Menez and co-workers (37, 162) for polypeptide antagonists of K channels. Thus, although κ-PVIIA has no obvious sequence homology to polypeptide toxins from other venomous animals that interact with voltage-gated K channels, there appear to be convergent functional features in diverse K channel polypeptide antagonists. Several peptides containing this structural feature have been identified from different venomous organisms, demonstrating that peptides with quite different cysteine backbones that interact with K channels can all have the dyad motif.

2. κA- and κM-conotoxins

The κA- and κM-conotoxins are structurally unrelated to each other; κA-conotoxins are *O*-glycosylated peptides that belong to the A-superfamily, while κM-conotoxins have a disulfide framework similar to the μ-conotoxins and belong to the M-superfamily (see Fig. 2).

The first peptide of the κA-conotoxin family was identified from the venom of the fish-hunting cone snail *Conus striatus*. This peptide, κA-conotoxin SIVA, causes spastic paralytic symptoms when injected into mice. The disulfide arrangement of κA-SIVA is similar to the pharmacologically distinct αA-conotoxins. Electrophysiological tests on diverse preparations provide evidence that κA-SIVA blocks K channels. Recordings from frog cutaneus pectoris muscle and principal neurons from frog sympathetic ganglion reveal that this peptide induces repetitive activity in these cells. Furthermore, *Shaker* channels expressed in *Xenopus* oocytes are blocked by micromolar concentrations of κA-SIVA. However, the molecular identity of the vertebrate high-affinity K channel target of this peptide has not yet been identified. Interestingly, it was shown that κA-SIVA in its active form contains one *O*-glycosylated serine at position 7, which was the first evidence for *O*-glycosylation as a posttranslational modification in a biologically active conotoxin. A peptide called CcTx from *Conus consors*, which has the same cysteine scaffold as κA-SIVA, was suggested to activate neuronal voltage-gated Na channels at resting membrane potential (101). If confirmed, this might indicate that similar peptides may act on different pharmacological targets, but a more thorough analysis of the κA-conotoxins and related peptides needs to be carried out.
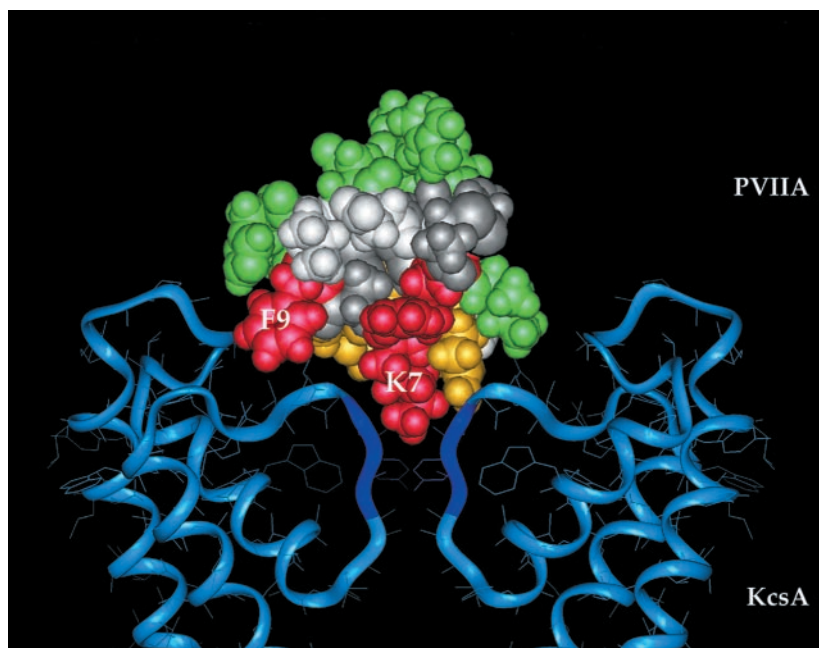

FIG. 3. Hypothetical docking orientation of κ-conotoxin PVIIA on the outer vestibule of the KcsA K channel pore. The two marked residues of the peptide, K7 and F9, comprise a dyad motif that is a general feature of polypeptidic toxins targeted to K channels. All residues colored red are major determinants of binding affinity, yellow residues make a measurable contribution, and green residues do not directly interact with the K channel blocked by κ-PVIIA. The *Shaker* K channel sequences have been overlaid on the KcsA crystal structure determined by McKinnon and co-workers (shown in blue) (39).

A new family of conotoxins, the κM-conotoxins, has the same class III scaffold as the μ- and ψ-conotoxins, but an entirely different target specificity: voltage-gated K channels (55). The first peptide belonging to this family, κM-RIIIK, was cloned from the venom duct of *Conus radiatus*. Unlike the structurally related μ-conotoxins, the peptide had no effect when tested on Na channels expressed in *Xenopus* oocytes. However, κM-RIIIK blocks *Shaker* K channels with an IC_{50} of ~1 μM and has an even higher affinity for TSha1, a *Shaker* homolog K channel from trout (IC_{50} ~20 nM).

The interaction of the peptide with the ion channel is a bimolecular reaction. Mutations of residues within the pore of the *Shaker* channel drastically affect the affinity of κM-RIIIK, indicating that like κ-conotoxin PVIIA, the peptide interacts with the ion channel pore. These results show that conotoxins with similar cysteine frameworks can have entirely different pharmacological properties. κM-RIIIK does not contain any phenylalanine or tyrosine present in other K channel-targeted peptides as part of the functional dyad, and it will be interesting to see whether a dyad motif is important for block of K channels by this conopeptide.

At least three additional families of conotoxins have been identified that are likely to be targeted to voltage-activated K channels (unpublished results). Interestingly, these peptides have entirely different cysteine connectivity from the three families above, which opens the possibility for novel polypeptidic ligands for investigating K channel structure and function. One group is peptides belonging to the I-superfamily (see Fig. 2); evidence that the family is extremely diverse has been obtained (85). Recently, an I-superfamily peptide (called κ-BtX) was shown to upmodulate the Ca- and voltage-sensitive BK currents measured from rat adrenal chromaffin cells and did not affect other voltage-gated channels (54), and another member of the I-superfamily designated VcTx was shown to inhibit K_v1.1 and K_v1.3 subtypes, but not K_v1.2 (88).

D. Ca Channel-Targeted ω-Conotoxins

Ca signaling is involved in a great variety of different physiological processes including neurotransmitter release. Voltage-gated Ca channels, which mediate the Ca influx in response to depolarization, are heteromeric protein complexes with four or five different subunits. The observed physiological and pharmacological diversity of Ca channels is mainly due to the properties of the pore-forming α_1-subunits. Based on their different physiological and pharmacological properties, voltage-activated Ca channels have been categorized into L-, N-, P-, Q-, R-, and T-type channels. More recently, a standardized, more genetically derived nomenclature has been proposed for Ca channels, which mainly adopts the system originally developed for K channels. According to this nomenclature, the Ca_v1 family includes the channels that mediate L-type Ca currents; the Ca_v2 family includes the channels which mediate the P/Q-type, N-type, and R-type Ca currents; and the Ca_v3 family includes the channels that mediate T-type Ca currents.

Conotoxins that target Ca channels were among the first conotoxins characterized and are the peptides from *Conus* venoms most intensively used in neuroscience research. The selective inhibition of different Ca channels specifically located at presynaptic endings was made possible with the discovery of ω-conotoxins. To date, ω-conotoxins have been largely identified from fish-hunting cone snails, although it seems likely that worm- or mollusc-hunting *Conus* species have developed an equally rich repertoire of peptides to target voltage-gated Ca channels. A peptide that inhibits molluscan Ca channels that corresponds to the L-type mammalian subtype has been described (51). The venoms of fish-hunting *Conus* that have been systematically studied will typically have multiple ω-conotoxin isoforms with major differences in their amino acid sequences. These are likely to be functionally selected for targeting different Ca channels. This was clearly demonstrated in *Conus magus* venom (68, 141); one peptide, ω-conotoxin MVIIA, is highly specific for N-type calcium channels ($Ca_v2.2$), while another peptide from the same venom, ω-conotoxin MVIIC, preferentially targets P/Q channels ($Ca_v2.1$). The structure of several ω-conotoxins has been solved. A characteristic feature of ω-conotoxins is their high content of basic amino acid residues that are known to play an important role for the inhibition of Ca channels (132). Besides these positive charges, it is known that a tyrosine residue (Tyr-13 in ω-MVIIA and ω-MVIIC) is important for the binding to $Ca_v2.2$ and $Ca_v2.1$ channels (90, 102, 134).

Several ω-conotoxins have been identified and functionally characterized, and there is an extensive literature on these peptides (for overviews, see Refs. 43, 122, 147). Since this field has previously been reviewed comprehensively, the reader is referred to these reviews for the earlier literature on ω-conotoxins. The sequences of the most widely used ω-conotoxins are shown in Table 2.

The therapeutic potential of ω-conotoxins that specifically target N-type Ca channels ($Ca_v2.2$) is an important application of conopeptide research. One of these peptides, ω-conotoxin MVIIA from *C. magus* (141), has been through extensive human clinical trials and was given "approvable" status for the treatment of intractable pain by the United States Food and Drug Administration (127, 138). This peptide has been provided with a generic name, ziconotide, and a commercial name, Prialt, by its developer, Elan Pharmaceuticals. Another peptide, ω-conotoxin CVID from *Conus catus* (103), is being explored as an antinociceptive agent (173); it has been given

TABLE 2.*Conopeptides targeted to K and Ca channels*

Peptide	*Conus* Species	Sequences	Reference Nos.
K channel-targeted peptides			
κ-Conotoxin PVIIA	*C. purpurascens*	CRIONQKCFQHLDDCCSRKCNRFNKCV	169
κM-Conotoxin RIIIK	*C. radiatus*	LOSCCSLNLRLCOVOACKRNOCCT*	55
κA-Conotoxin SIVA	*C. striatus*	ZKSLVP$VITTCCGYDOGTMCOOCRCTNSC*	30
Ca channel-targeted peptides			
ω-Conotoxin GVIA	*C. geographus*	CKSOGSSCSOTSYNCCRSCNOYTKRCY*	146
ω-Conotoxin MVIIA	*C. magus*	CKGKGAKCSRLMYDCCTGSCRSGKC*	140
ω-Conotoxin MVIIC	*C. magus*	CKGKGAPCRKTMYDCCSGSCGRRGKC*	68
ω-Conotoxin CVID	*C. catus*	CKSKGAKCSKLMYDCCTGSCSGTVGTC*	1

$, *O*-glycosylated Ser residue; Z, pyroglutamtamic acid; O, *trans*-hydroxyproline; * COOH-terminal amidation.

the pharmaceutical designation AM336 and was slated for clinical development by Amrad, an Australian pharmaceutical company.

The antinociceptive potential of $Ca_v2.2$-targeted ω-conotoxins has two important scientific facets. The first is that in the mammalian spinal cord, the synapses with incoming C-fibers that carry nociceptive signals are particularly sensitive to block by these ω-conotoxins; autoradiographic studies revealed an enrichment in $Ca_v2.2$ channels at these sites in the dorsal horn. Furthermore, because these peptides are antagonists of voltage-gated ion channels, in contrast to opioids which are agonists of G protein-coupled receptors, they do not have the problems encountered upon continued exposure of the latter receptors to agonists that cause downregulation. This downregulation is the basis for development of tolerance to opioid drugs that occurs in patients; this does not occur in patients treated with the peptide. Thus $Ca_v2.2$-targeted conopeptides are envisioned to be useful in clinical situations where opioid drugs are no longer (or have never been) effective.

E. State Dependence of Block: Transitions Between States

Because voltage-gated ion channels must necessarily undergo conformational changes to carry out their functions, the binding of pharmacologically active substances may differ depending on the state of the ion channel. Such state or use dependence was previously demonstrated; for example, the block of Na channels by TTX or STX has a tonic component and a phasic component, which depends on the activation of the Na channels (e.g., Refs. 7, 14, 23, 24, 44, 108, 159, 160). However, few investigations on state dependence for polypeptide antagonists of voltage-gated ion channels have been reported.

Recently, the binding of κ-PVIIA to *Shaker* K channels was investigated, primarily using electrophysiology. The observed changes in current kinetics in the presence of the toxin were explained by differences in both the steady-state affinity as well as binding kinetics to the open and closed state of the channel (179). Furthermore, it was shown that κ-PVIIA binding to closed but not to open *Shaker* channels depended on the extracellular K concentration: an increase leads to a reduction of the steady-state affinity to the closed state (see Fig. 4). The observed acceleration of κ-PVIIA dissociation from open channels in the presence of physiological levels of extracellular K concentration was attributed to the voltage-dependent occupancy by K ions of a site at the outer end of the conducting pore. Occupancy of this site by external cations most likely antagonizes on-binding to closed channels, whereas the apparent competition disappears in open channels if the competing cation can move along the pore. Some of the experimental results that led to this picture are shown in Figure 4.

The investigation of the effects of the ionic milieu on κ-PVIIA binding further revealed that binding to the closed channel is independent of the intracellular cation. Additionally, it was found that equally permeant cations may have quite different occupancy configurations within the pore permeation pathway (11). Studies on mutant *Shaker* channels in which C-type inactivation is accelerated demonstrated that κ-PVIIA binding and C-type inactivation are mutually exclusive (94). This is functionally significant, since under certain conditions, the binding of κ-PVIIA leads to an increase in the evoked currents (instead of the block of K currents that would generally be expected for this peptide).

These results show that binding of κ-PVIIA to *Shaker* K channels depends on and may affect the conformational state of the channel in a way that is functionally, and thereby physiologically, relevant. This analysis suggests that a detailed study of the interaction of substances blocking ion channels with permeant ions may provide important information regarding properties of the ion-conduction pathway and might add complementary data to the recent advances made on the knowledge about the structure of the permeation pathway of K channels.
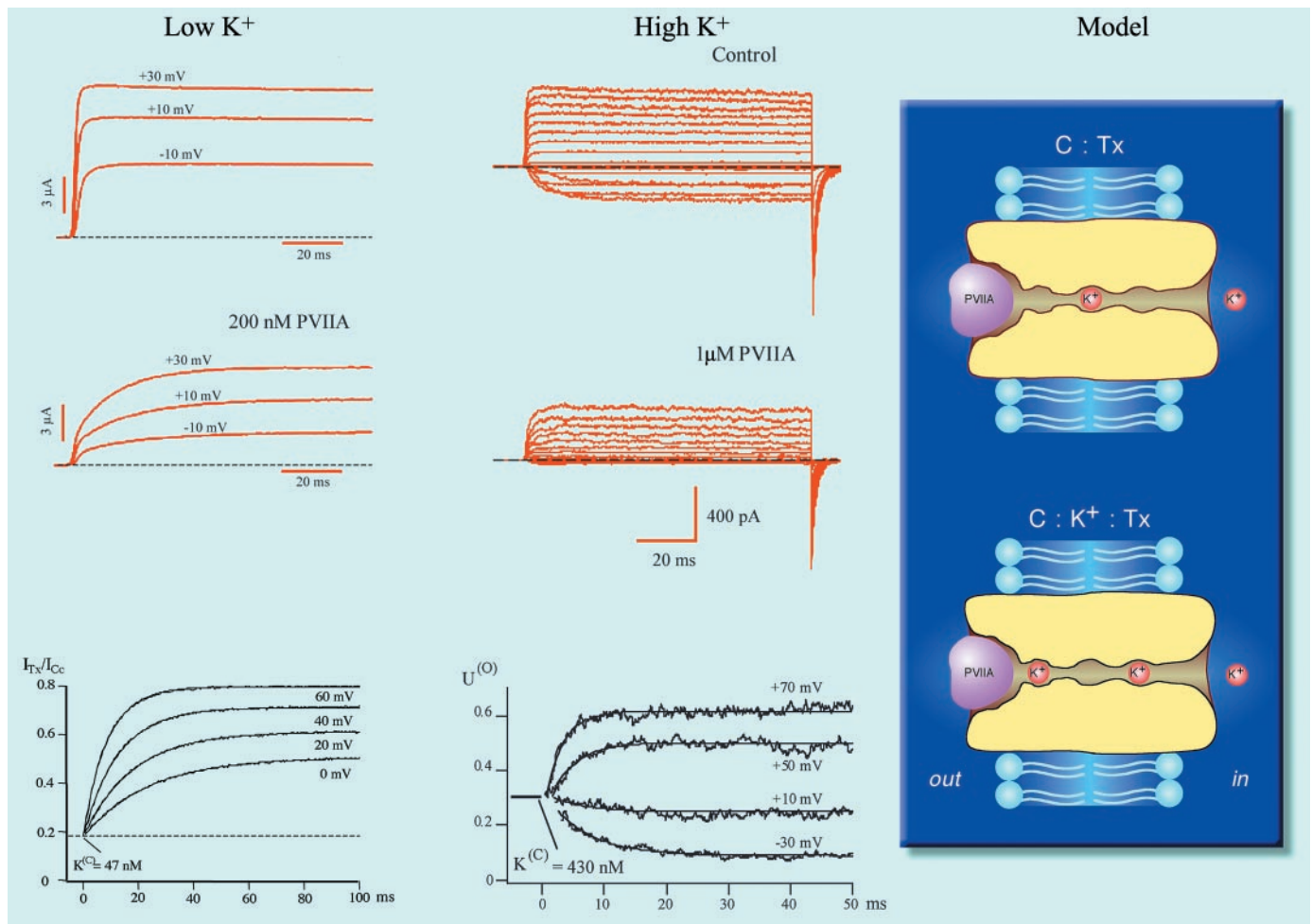
FIG. 4. The block of κ-conotoxin PVIIA is state dependent. *Left panels*: the effect of κ-PVIIA on *Shaker*-Δ6–46 channels. Two-electrode voltage-clamp recordings from an oocyte expressing *Shaker*-Δ6–46 channels before (*top*) and after (*middle*) the bath application of 200 nM κ-PVIIA in the presence of 2.5 mM extracellular K. The toxin causes a strong reduction of the early currents but has a much smaller effect on the steady-state conductance activated by step depolarizations. Current ratios, or unblock probabilities, U(t), for the indicated voltage steps are shown at the *bottom*. At the half-activation time of the control responses, U is ~0.19 for all voltages and corresponds to a toxin dissociation constant from closed channels, $K^{(C)}$ = 47 nM. Thereafter, U(t) is well fitted by a single-exponential relaxation (solid lines), indicating a voltage-dependent relaxation of the binding to a new equilibrium. *Middle panels*: currents of *Shaker*-Δ6–46 channels recorded from an outside-out patch in symmetric 115 mM K without (control) and with 1 μM κ-PVIIA in the bath. The current ratios (*bottom panel*) reveal that the dissociation constant from closed channels under those conditions is $K^{(C)}$ = 430 nM. For the different test voltages applied, the block either increases (−30 to +10 mV) or decreases (+50 and +70 mV). *Right panels*: model of the κ-PVIIA block. When the outermost K binding site within the ion channel pore [most likely site 1 within the selectivity filter (130, 195)] is occupied by a potassium ion (C:K:Tx), a bound κ-PVIIA molecule experiences a strong repulsion. This repulsion is much weaker when this site is not occupied by K (C:Tx). The probability that this site is occupied will be high for high extracellular K concentrations, but also during K flow through the channel after channel opening in the presence of low extracellular K. [Modified from Terlau et al. (179).]

State-dependent binding has recently been shown for binding of κM-RIIIK to its target K channels (55); peptide neurotoxins from other organisms have been demonstrated to also interact in a state-dependent fashion (5). This opens up the possibility that the state dependence might be a common feature of the interaction of biologically active substances with voltage-gated ion channels.

III. *CONUS* PEPTIDES TARGETED TO LIGAND-GATED ION CHANNELS

A. Overview

Ligand-gated ion channels are proteins that mediate fast synaptic transmission (for an overview, see Kandel et al., Ref. 87). Because many of these membrane-bound

proteins have been cloned, they are grouped according to their structural and functional similarities. One major group of ligand-gated ion channels, all belonging to the same gene superfamily, are those activated by acetylcholine, serotonin, GABA, or glycine. The functional channel protein complexes are composed of five subunits, with each subunit containing four transmembrane helices. Besides their ligand specificity, these proteins differ in their selectivity for permeant ions. The other gene superfamily of ligand-gated ion channels is the glutamate receptors, usually subdivided into N-methyl-D-aspartate (NMDA) and non-NMDA (kainate/AMPA) receptors. A third family of ligand-gated ion channels involved in synaptic transmission at certain synapses is the ATP receptors.

Conus peptides targeted to three different families of ligand-gated ion channels have been identified. *Conus* peptides that target the major ligand-gated ion channel superfamily are ubiquitously distributed in *Conus* venoms. One peptide has been shown to target the 5-hydroxytryptamine (5-HT$_3$) receptor (48), with a large number known to target nicotinic acetylcholine receptors (124). Competitive nicotinic agonists are particularly well represented among *Conus* peptides, but noncompetitive nicotinic antagonists have also been characterized. An unusual family of *Conus* peptides, the conantokins, are antagonists of NMDA receptors, a subclass of the glutamate receptor superfamily. These peptides are biochemically distinctive in their high content of the modified amino acid γ-carboxyglutamate and lack of Cys residues.

The most widely distributed of the nicotinic antagonists are the α-conotoxins, multiple representatives of which are expressed in the venom ducts of most, if not all, *Conus* species. Thus we expect that the total number of α-conotoxins in *Conus* venoms will be >>1,000 different peptides. As discussed below, α-conotoxins are highly subtype-selective nicotinic antagonists and are proving to be valuable pharmacological reagents for discriminating between closely related neuronal nicotinic acetylcholine receptor isoforms.

The endogenous functions of *Conus* peptides targeted to various ligand-gated ion channels can be speculated on. The major nicotinic acetylcholine receptor antagonists found in a venom are usually those used to disrupt neuromuscular transmission to paralyze prey; these are presumably targeted to the main nicotinic acetylcholine receptor subtype(s) present at the neuromuscular junction of the prey. This is clearly the case for fish-hunting (piscivorous) cone snails, where the quantitatively most abundant peptides of the α-conotoxin family in the venom are paralytic to fish (and other vertebrates). The endogenous functions of nicotinic antagonists that are not targeted to the neuromuscular nicotinic acetylcholine receptors (nAChR) are far less clear. It has been hypothesized that a subset of these may be involved in suppressing the fight-or-flight response of the prey (177).

Several neuronal nAChR subtypes are present in the autonomic circuitry of vertebrates.

An entirely different set of functions has been hypothesized for the glutamate and 5-HT$_3$ receptor antagonists (conantokins and σ-conotoxin). It has been suggested that the snails from which these have been isolated use the peptides as part of the nirvana cabal whose overall function is to deaden the sensory circuitry of the prey (140). This elicits a sedated, opium den-like state, making the prey easier to capture, and to handle once captured. The use of a nirvana cabal is clearest in net-hunting piscivorous species (that probably try to capture schools of small fish in the wild).

B. α-Conotoxins and nAChRs: Matching a Family of Conopeptides to a Family of Ion Channel Targets

Of all conopeptide families, the diverse targeting specificity of the α-conotoxins is the best characterized at present. The α-conotoxins were among the first conopeptides discovered; however, the first family members characterized were all targeted to the nicotinic receptor subtype at the neuromuscular junction (see Ref. 124 for a review). Beginning with the discovery of α-conotoxin ImI, McIntosh et al. (125) have characterized individual α-conotoxins that are selectively targeted to diverse nicotinic receptor subtypes. Some α-conotoxins of potential importance for basic neuroscience research or with potential for clinical development are shown in Table 3.

Nicotinic receptors belong to the major ligand-gated ion channel superfamily. Like all superfamily members, functional nicotinic receptors are believed to be pentameric complexes. For a subset of nicotinic receptors, a single type of subunit can assemble into a functional homopentameric receptor; this is characteristic of α_7-, α_8-, and α_9-subunits. Although such homomeric receptor complexes are a minority of total nAChR diversity in vertebrate systems, this may not be the case in many invertebrate phyla, where the majority of nicotinic receptor subunit genes appear to be most closely related to the vertebrate α_7-, α_8-, and α_9-subunits that are known to form homomeric complexes.

Most vertebrate nicotinic receptors are heteromeric arrays, typically consisting of two α-subunits and three non-α-subunits. There may be between two and four different types of subunits comprising the native functional heteromeric nicotinic receptor complex. The ACh ligand sites (also the binding site for other agonists) are located at interfaces between α- and non-α-subunits, and two ACh must bind before gating of the ion channel from a closed to an open state can occur. A wide variety of competitive antagonists that compete for binding to the ACh site have been characterized (4, 117). The classical competitive

TABLE 3. *Natural α-conotoxins with potentially useful targeting specificity or reported clinical promise*

Peptide	Nicotinic Receptor Specificity	Sequence	*Conus* Species	Reference Nos.
α-MI	$\alpha_1\delta >> \alpha_1\gamma$	GRCCHPACGKNYSC*	*C. magus*	77, 119, 170
α-EI	$\alpha_1\gamma \approx \alpha_1\delta$	RDOCCYHPTCNMSNPQIC*	*C. ermineus*	114
α-AuIB	$\alpha_3\beta_4$	GCCSYPPCFATNPDC*	*C. aulicus*	110
α-MII	$\alpha_3\beta_2 \approx \alpha_6\beta_2\beta_3$	GCCSNPVCHLEHSNLC*	*C. magus*	16
α-PIA	$\alpha_6\beta_2\beta_3 > \alpha_3\beta_2$	RDPCCSNPVCTVHNPQIC*	*C. purpurascens*	6; Dowell et al., unpublished data
α-ImI	α_7	GCCSDPRCAWRC*	*C. imperialis*	125
α-ImII	α_7*	ACCSDRRCRWRC*	*C. imperialis*	45
α-PnIA	$\alpha_3\beta_2$	GCCSLPPCAANNPDYC*	*C. pennaceus*	50
α-PnIB	α_7	GCCSLPPCALSNPDYC*	*C. pennaceus*	111
"Vc1.1"	(?)	GCCSDPRCNYDHPEIC*	*C. victoriae*	107

For nicotinic receptor specificity, all receptor subtypes are for mammalian nAChRs. α-MI belongs to the α3/5-subfamily, α-ImI and α-ImII to the α4/3-subfamily, and all other peptides to the α4/7-subfamily (see text). * Amidated COOH terminus.

nicotinic antagonists are the well-known snake toxin from the banded krait, α-bungarotoxin, and the South American Indian poison arrow alkaloid, curare. These were historically significant reagents in neuroscience, essential in the initial characterization of the muscle nicotinic receptor subtype. However, relatively few antagonists that specifically target other nicotinic receptor subtypes were previously known; only in recent years, with the systematic characterization of the α-conotoxins, has this void begun to be filled.

α-Conotoxins target all of the classes of nicotinic receptors described above. Within this widely distributed conopeptide family, some subfamily specialization can be discerned. Some of the α-conotoxin subfamilies are narrowly distributed among a small group of related *Conus* species, such as the α3/5-conotoxin subfamily, which are major α-conotoxins found in many fish-hunting *Conus* venoms. These have the primary sequence motif -CCX$_3$CX$_5$C- and are all paralytic conotoxins, presumably targeted to the muscle nicotinic receptor subtype in the fish prey of these *Conus* species. A comprehensive discussion of these peptides was presented in the review by McIntosh et al. (124).

When tested on mammalian nicotinic receptors, many α3/5-conotoxins appear to be highly specific for only one of the two ligand binding sites. For example, α-conotoxin MI has an ~10^4 preference for the binding site at the α_1/δ-interface vs. the ligand site at the α_1/γ-interface. The amino acid determinants on the δ-subunit that confer higher binding affinity for α-conotoxin MI have been identified (170). Additionally, it is notable that the best-characterized α3/5-conotoxins, α-conotoxins MI and GI, are much more specifically targeted to the muscle nAChR subtype than other muscle nAChR ligands. Both peptides inhibit the muscle subtype preferentially by several orders of magnitude; in contrast, α-bungarotoxin inhibits both the muscle and α_7-subtypes, while curare also inhibits a variety of neuronal nAChRs in addition to the muscle subtype. As a group, the α3/5-conotoxins are more

discriminating for the muscle nAChR than any other ligands known, and many of these peptides discriminate between the two ligand binding sites on the muscle nAChR.

Multiple α3/5-conotoxins encoded by different genes can often be found in a single venom, all of which are paralytic to fish (an example is *Conus striatus* venom, which contains α-conotoxins SI, SIA, and SII, which though divergent in sequence are all paralytic α3/5-conotoxins). The reason for having multiple peptides apparently all acting on nicotinic receptors at the fish neuromuscular junction is not understood and implies some yet-undefined complexity in the neuromuscular nAChRs of the fish prey of this cone snail. All α3/5-conotoxins characterized so far are potently paralytic when injected into fish; however, their potency when tested on higher vertebrates appears to be an unselected variable. Some α3/5-peptides are extremely potent inhibitors of the mammalian muscle subtype (such as α-conotoxins MI and GI), while others have much decreased affinity for the mammalian receptor (such as α-conotoxin SI).

Another subfamily of the α-conotoxins is the α4/3-subfamily, in which the sequence motif is -CCX$_4$CX$_3$C-. These α-conotoxins are restricted to a group of related species known to eat amphinomids, which are polychaete worms characteristically armed with defensive bristles (trivially known as fireworms). The best studied of the α4/3-conotoxin subfamily is two peptides from *C. imperialis*, α-conotoxins ImI and ImII, which have both been shown to inhibit homomeric nicotinic receptors composed exclusively of α_7-subunits. It is possible that just as the α3/5-subfamily is specialized for the muscle subtype of nicotinic receptors, the α4/3-subfamily may be specialized to target homomeric nicotinic receptors, although at the present time, these conopeptides are less well characterized.

The interactions of α-conotoxin ImI with the α_7-nicotinic receptor have been investigated (150, 152). The

second peptide from *C. imperialis*, α-conotoxin ImII, is largely identical in sequence to α-conotoxin ImI (9 out of 12 amino acids identical). Not surprisingly, it was found that α-conotoxin ImII also targeted the α_7-subtype. A most unexpected result, however, was that α-conotoxin ImII did not act at the competitive ligand site that is the high-affinity target of both α-bungarotoxin and α-conotoxin ImI (45).

It was shown that a specific proline residue (Pro-5 in α-conotoxin ImI) was required for targeting these peptides to the competitive ligand site; α-conotoxin ImII lacks this Pro-5 residue; instead, there is Arg substitution at this locus. The lack of proline apparently results in α-conotoxin ImII targeting an entirely different site on the α_7-receptor; however, the α-conotoxin ImII binding site has not yet been localized. Thus, although most α-conotoxins are likely to be competitive antagonists, the results with α-conotoxin ImII indicate that at least for those targeted to homomeric nicotinic receptors, not all α-conotoxins function as competitive antagonists. It is worth noting that most α-conotoxins have the proline residue critical for competitive antagonism in α-conotoxin ImI (see Table 3); the small minority of α-conotoxins (such as α-ImII) that lack this highly conserved Pro residue (see Table 3) are potential candidates for acting through a different site.

The largest and most widely distributed subfamily of the α-conotoxins are the α4/7-conopeptides (motif: -CCX$_4$CX$_7$C-). Although each individual conopeptide is probably very specifically targeted, as a group, targets of this subfamily comprise every class of nicotinic receptor, including the muscle subtype (example, α-conotoxin EI), homomeric nicotinic receptor subtypes such as α_7 (example, α-conotoxin PnIB), and heteromeric neuronal nicotinic subtypes (examples, α-conotoxin MII and α-conotoxin AuIB); the amino acid sequences of these peptides are shown in Table 3.

A very few amino acid substitutions in α4/7-conotoxins can apparently cause a shift in targeting specificity and affinity. α-Conotoxins PnIA and PnIB from the venom of *Conus pennaceus* are an example; these peptides differ in sequence by only two amino acids, yet one preferentially targets the α_7-receptor, and the other the $\alpha_3\beta_2$-nAChR subtype. In an analysis of which of the two amino acid differences was important (111), it was discovered that a chimeric peptide between α-conotoxins PnIA and PnIB had even higher affinity for the α_7-receptor than did either of the original natural peptides (the complementary chimera had poorer affinity than either natural peptide). Thus changes in critical amino acid loci on the α4/7-conotoxins can dramatically change binding affinity and pharmacological specificity.

C. α-Conotoxins and Subtype Selectivity

The work done on the α-conotoxins and nAChRs raises issues generally relevant to conotoxin subtype selectivity that are discussed in this section, as well as mechanisms that may underlie α-conotoxin discrimination between different nAChR subtypes. The standard approach used to identify α-conotoxins that have nAChR subtype selectivity is to express a single nAChR isoform in a heterologous system such as *Xenopus* oocytes or HEK cells, and to screen crude *Conus* venoms for effects on nAChR function. Venom that shows strong activity for a specific nAChR subtype is fractionated, and the active peptide is purified and characterized. Alternative approaches to obtaining subtype-specific α-conotoxins are to synthesize peptides predicted by cDNA clones and to test the activity of such peptides on a panel of nAChR subtypes; a related approach (46) is to screen combinatorial libraries derived from native α-conopeptides.

A possible biological rationale for why conopeptides have evolved to be highly subtype selective is given in section I. Basically, each conopeptide has a physiologically relevant target in the prey, predators, or competitors of that *Conus* species. The ion channels used as an assay for conopeptide purification and characterization are invariably from a mammalian source; these mammalian ion channels are not the actual target of any conopeptide. The surprising degree of selectivity observed for mammalian ion channel subtypes probably arises for a variety of different reasons.

The molecular target of the conopeptide may be highly conserved, such that the mammalian isoform is not distinguishable to the conopeptide from its true target. This is especially likely if an α-conotoxin is purified from a fish-hunting cone snail venom; since vertebrate nicotinic receptors are conserved, it is quite possible that a mammalian nicotinic receptor isoform is close in sequence at the conopeptide binding site to the corresponding homolog in fish. In this case, the α-conotoxin retains high affinity for a mammalian nicotinic receptor subtype because it is closely similar to its physiologically relevant target.

However, some of the most useful and selective α-conotoxins are isolated from snail-hunting and worm-hunting *Conus* species. Close sequence similarity of snail or worm subtypes to mammalian subtypes is less likely, since the evolutionary distance to mammalian subunits is much greater. Not surprisingly, the affinity found for mammalian receptors is generally not as high as is found with *Conus* peptides from fish-hunting cone snail venoms. Furthermore, the discrimination between subtypes may not be as striking; even if the peptide did discriminate strongly between the target and closely related subtypes present in the relevant organism, one would not neces-

sarily expect the same degree of discrimination for nicotinic receptors in a distantly related nervous system.

Surprisingly however, some peptides from the venoms of *Conus* species that prey on invertebrates retain very high selectivity for particular mammalian receptor subtypes, and some of these exhibit an extremely broad phylogenetic range. Thus α-conotoxin ImI, from the venom of a worm-hunting species, discriminates between homomeric and heteromeric nAChR subtypes in a wide variety of phylogenetic systems. In molluscs, this peptide will even inhibit nAChRs that gate anions instead of cations (89). In all systems, the peptide appears to affect only a single, or small subset, of nAChRs, always receptor subtypes that are rapidly desensitizing (124). The peptide could be recognizing some conserved feature of rapidly desensitizing nAChRs, and by being targeted to such a conserved feature, may not be highly sensitive to the general background of amino acid sequence changes that have taken place across the different phyla.

At a molecular level, the precise mechanism of molecular recognition is not well understood. The use of double mutant cycling analysis has led to a considerable body of work that identifies amino acids on the α-conotoxin interacting with specific amino acids on nicotinic receptor subunits (12, 150–153, 176). One mechanism proposed for the high subtype selectivity is that α-conotoxins really have two pharmacophores, for two distinct sites on the receptor complex; this is known as the Janus ligand hypothesis (137, 144). In essence, by having two recognition sites, an α-conotoxin can be specific for an interface between two particular nAChR subunits.

The experimental data supporting the Janus ligand hypothesis comes from studies with α-conotoxin MII and its interaction between a high-affinity target nAChR subtype (the $\alpha_3\beta_2$-receptor) and another subtype, the $\alpha_3\beta_4$-receptor, for which the peptide has a four orders of magnitude lower affinity. A striking kinetic result was obtained (15), although the affinities and on-times for the two nAChR subtypes differed by $\sim10^4$, it was found that the k_{off} values for the two nAChR subtypes were essentially identical. These data led to the hypothesis that a site on the β_2-subunit allowed very rapid docking of the peptide onto the receptor complex, with the α-conotoxin subsequently transferred to a different site on the α_3-subunit (therefore present in both $\alpha_3\beta_2$- and $\alpha_3\beta_4$-complexes); this accounts for the identical k_{off} values observed. Because of the absence of the docking site from the β_4-subunit, the on-time for the $\alpha_3\beta_4$-subtype is 10^4 slower. This hypothesis is not a unique explanation for the experimental data; other models involving conformational changes in either the peptide, the nAChR, or in both would also fit the observed data.

Although much remains to be done to understand α-conotoxin subtype selectivity, the broad picture that has emerged suggests that the disulfide framework of α-conotoxins is "preoptimized" for interaction with ligand sites on nAChRs. Thus this becomes the most likely gene family to evolve novel specificity for nAChRs. In the course of the last 50 million years, as new *Conus* species were evolving, whenever an ecological scenario arose so that antagonism of some nAChR isoform might help capture new prey more efficiently (or fend off new predators or competitors), this gene family was consistently the most likely to yield the novel nicotinic antagonist with the appropriate targeting specificity for the incipient species to survive and flourish.

The binding determinants that contribute significantly to the high affinity of α-conotoxins have been elucidated for a number of conopeptide:nAChR pairs. The number of nicotinic receptor sequences available will continue to increase, and we can expect more and more α-conotoxin binding sites to be defined. As the relevant bioinformatics database expands, a "designer" strategy for α-conotoxins highly specific for particular subtypes will become increasingly feasible. Predicting which amino acid substitutions on an α-conotoxin will confer greater or lesser affinity for a particular nicotinic receptor subtype should become increasingly accurate. Thus the systematic collection of sequence and specificity-of-binding information for the α-conotoxin:nicotinic receptor pairs could lead to the systematic development of a set of designer ligands for each nAChR isoform, a development that should constitute a leading edge in the field of protein-protein interactions. Such a technology platform would have promising applications for both therapeutics and basic neuroscience (see sect. IIID).

D. Defining Molecular Isoforms of Nicotinic Receptors: Combining α-Conotoxins With Knockout Mutants

The potential molecular complexity of nicotinic receptors in the central nervous system makes the functional determination of their roles in a given central nervous system circuit or region a challenging problem. One productive approach is to combine subtype-specific α-conotoxins with knockout mutants. The combination is particularly powerful for targets such as nAChRs because knockout mutants of a particular subunit would, in principle, cause a lesion of multiple functional nAChR complexes; for example, a knockout of the α_3-nAChR subunit would delete both the $\alpha_3\beta_4$- and the $\alpha_3\beta_2$-subtypes as well as any more complex combinations that contain α_3-subunits.

A combination of subtype-specific α-conotoxins with knockout mice has recently been used to establish that one of the major subtypes involved in nicotinic modulation of dopamine release in the striatum is a receptor complex with $\alpha_6\beta_2\beta_3$-subunits. It had been previously

established that α-conotoxin MII inhibited a significant fraction ($\sim$40%) of nicotine-evoked dopamine release (99); in an α_4-knockout, $\sim$90% inhibition by α-conotoxin MII was observed. In α_6-knockout mice, all binding of α-conotoxin MII in the striatum was abolished (20); in contrast, in α_3-subunit knockouts, expression of the great majority of radiolabeled α-conotoxin MII binding sites was not affected, suggesting that $\alpha_3\beta_2$-nicotinic receptors probably do not play a major role. The only region where α_3-dependent α-conotoxin MII binding was found at significant levels was in the habenulointerpeduncular tract (189). Additionally, it has been found subsequently that both β_2- as well as β_3-knockout mice (3) have much reduced radiolabeled α-conotoxin MII sites expressed in the striatum. Together, these experiments suggest that a nicotinic receptor complex containing three subunits, $\alpha_6\beta_3\beta_2$, is the major α-conotoxin MII-sensitive molecular isoform that modulates dopamine release. Immunoprecipitation experiments suggest even more complex combinations, such as $\alpha_4\alpha_6\beta_2\beta_3$, may make a minor contribution (196). The functional role of α_6-subunits in nicotine-evoked dopamine release in the striatum has been confirmed pharmacologically by the use of newly developed α-conotoxins, such as α-conotoxin PIA that discriminate between α_6- and α_3-containing nAChRs (6).

A number of caveats, which apply to all conotoxins, should be raised regarding the use of α-conotoxins as pharmacological reagents. Conotoxins can clearly be used both as ligands for binding (and therefore as reagents for autoradiography or microscopy) as well as antagonists in functional experiments. It should not be automatically assumed that binding corresponds to function; an α-conotoxin could bind to a complex, but not functionally inhibit that receptor complex. An additional complexity is that although the primary binding site is an interface between two subunits, the presence of other subunits in the complex could affect the affinity of the peptide for the nAChR. Thus, even if an α-conotoxin has demonstrable functional antagonism for a particular nAChR using an expression system such as oocytes as an assay system, a discrepancy may be found when the α-conotoxin is tested in vivo. For example, the presence

in the complex of otherwise "silent" subunits such as α_5 or β_3 could have an effect on α-conotoxin affinity.

However, as more individual α-conotoxins are tested on native receptors, both their binding specificity and their functional activity become better defined. This information will be increasingly important for elucidating nicotinic receptor function, given the potential combinatorial complexity. Thus, in the caudate putamen alone, it has been suggested that there are five different molecular isoforms of nicotinic receptors (196), four of which are found on striatal dopaminergic terminals ($\alpha_4\beta_2$, $\alpha_4\alpha_5\beta_2$, $\alpha_4\alpha_6\beta_2\beta_3$, and $\alpha_5\beta_2\beta_3$) and two of which are found in nondopaminergic neuronal structures ($\alpha_4\beta_2$ and $\alpha_2\beta_4\beta_2$). A functional dissection of this plethora of subtypes in all of the different regions of the brain is the challenge that lies ahead, and the combination of subtype-specific α-conotoxins with knockout mice should be an attractive technology for meeting this challenge.

E. Other Conopeptide Families Targeted to Ligand-Gated Ion Channels

In addition to the α-conotoxins, which are discussed in the sections above, several other conopeptide families have been shown to target ligand-gated ion channels. These are shown in Table 4. Like α-conotoxins, two of these families, the αA-conotoxins and the ψ-conotoxins, are antagonists of nicotinic receptors. All peptides belonging to these families that have been characterized target the muscle subtype of nicotinic receptors. The αA-conotoxins have three disulfide bonds instead of the two found in α-conotoxins, but like the latter, they are competitive nicotinic antagonists (73, 76). However, they do not have the same high selectivity for the α_1/δ-ligand site that is characteristic of the α3/5-subfamily of conotoxins. Like the α3/5-subfamily of conotoxins, these appear to be a special adaptation of a relatively small group of fish-hunting cone snail species for efficiently paralyzing fish.

Another conopeptide family targeted to nicotinic receptors are the ψ-conotoxins; these are generally found in

TABLE 4.*Conopeptide families targeted to ligand-gated ion channels*

Family	Target	Example	Sequence	Reference Nos.
σ-Conotoxins	5-HT$_3$ receptor	σ-GVIIIA	GCTRTCGGOKCTGTCTCTNSSKCGCRYNVHPSGBGCGCACS*	48
α-Conotoxins	Nicotinic receptors	α-GI	ECCNPACGRHYSC*	59
αA-Conotoxins	Nicotinic receptors	αA-PIVA	GCCGSYONAACHOCSCKDROSYCGQ*	73
		αA-EIVA	GCCPYONAACHOCGCKVGROOYCDROSGG*	76
ψ-Conotoxins	Nicotinic receptors[+]	ψ-PIIIE	HOOCCLYGKCRRYOGCSSASCCQR*	165
Conantokins	NMDA receptors	Conantokin-G	GE$\gamma\gamma$LQγNQγLIRγKSN*	123
		Conantokin-T	GE$\gamma\gamma$VQKMLγNLRγAEVKKNA*	61
		Conantokin-R	GE$\gamma\gamma$VAKMAAγLARγNIAKGCKVNCYP	188
		Conantokin-L	GE$\gamma\gamma$VAKMAAγLARγDAVN*	83

γ, γ-Carboxyglutamate; B, 6-bromotryptophan. * Amidated COOH terminus. [+]Noncompetitive antagonist.

the same venoms that contain the αA-conotoxins. However, in contrast to most α- and αA-conotoxins, the ψ-conotoxins are noncompetitive antagonists of the muscle subtype of nicotinic receptors. So far, both of these families are relatively poorly characterized, although the structures of several αA-conotoxins and two ψ-conotoxins have been determined (63, 128, 165, 182, 183). Structurally, the ψ-conotoxins are most closely related to the μ-conotoxins that are Na channel blockers, and the κM-conotoxins that act on K channels; although the three conotoxin families affect different targets, all of them may effectively act by plugging the ion permeation pathway.

The only other receptor that belongs to the major ligand-gated ion channel superfamily for which a conopeptide has been defined is the 5-HT$_3$ receptor. An unusual conopeptide containing bromotryptophan, σ-conotoxin GVIIIA, was isolated and shown to be a high-affinity antagonist of the 5-HT$_3$ receptor (48). So far, this is the only peptidic toxin known to target this receptor. Many other peptides belong to the same gene superfamily as σ-conotoxin GVIIIA, but because of their sequence divergence, they appear unlikely to target the 5-HT$_3$ receptor.

A final group of conopeptides that target ligand-gated ion channels targets a different gene superfamily, the glutamate receptors. These unusual peptides, the conantokins, are antagonists of a subclass of glutamate receptors, the NMDA receptors. In contrast to the ligand-gated ion channel superfamily, glutamate receptors are believed to be tetramers, and in the NMDA subclass, the functional native receptors consist of two types of subunits, NR1 and NR2. So far, only one gene has been found to encode NR1 subunits, but several different splice variants have been found. In contrast, the four NR2 subunits, NR2A, NR2B, NR2C, and NR2D, are encoded by different genes.

The best-characterized conopeptide targeted to the NMDA receptor is conantokin-G, which was originally purified using a behavioral assay. The peptide put young mice into a sleeplike state (before its mechanism was elucidated, it was called the "sleeper peptide") but made older mice hyperactive (142, 145). Conantokin-G is a small, 17-amino acid linear peptide without disulfide bonds. The biochemical characterization of the peptide revealed five residues of the nonstandard amino acid γ-carboxyglutamate (Gla or γ). Gla is synthesized from glutamate by a vitamin K-dependent γ-glutamyl carboxylase; the discovery of Gla in conantokin-G established for the first time the presence of Gla in invertebrate systems (123). The posttranslational modification enzyme from *Conus* venom ducts that converts Glu to γ-carboxyglutamate has been cloned and expressed and shown to be highly homologous to the mammalian enzyme (9, 36). Because of the absence of disulfides in this peptide, it is the Gla residues that provide the structural framework for forming a helical conformation (22, 131, 154, 171).

The initial evidence suggesting that the peptide inhibited NMDA receptors was obtained using a neonatal rat cerebellum preparation; conantokin-G blocked the enhancement of cGMP induced by the addition of NMDA but had no effect on the kainate-induced elevation of the cyclic nucleotide (126). It was subsequently shown that the peptide inhibited NMDA-mediated currents in mouse brain mRNA-injected oocytes (62) and also blocked the NMDA-mediated increase in intracellular free Ca^{2+} using cerebellar granule cells in culture (61). These experiments also suggested that conantokin-G acts as a competitive antagonist. Recently, point mutations in the NMDA receptor that affect conantokin-G affinity have been identified (192). It has been established (100) that the glutamate binding site is located on the extracellular domains of NR2 subunits, while the homologous positions in the NR1 subunit interact with glycine, the coagonist for NMDA receptors. Mutations at these sites in the NR1a and NR2B subunits were evaluated, and only substitutions in the glutamate binding site of the NR2B subunit resulted in a significant increase in the apparent inhibition constant for conantokin-G (10- to 100-fold). Only very modest effects from mutations at the homologous positions in the NR1a subunit were observed. These results are consistent with a competitive mechanism at the glutamate binding site of the NR2B subunit, but not the glycine site on the NR1 subunit.

A competitive mechanism of antagonism at the glutamate site is supported by several other experimental observations. The suppression of NMDA-activated currents in oocytes by conantokin-G could be overcome by increasing the concentration of NMDA, but not by increasing the concentration of glycine (62). Furthermore, block by conantokin-G was not voltage dependent. However, a variety of experimental observations suggest that in addition to interacting competitively with the glutamate site, conantokin-G has a more complex interaction with the NMDA receptor. Thus conantokin-G inhibits spermine- and spermidine-stimulated MK801 binding to rat forebrain membranes in a noncompetitive manner (172). Furthermore, it was observed by Mena et al. (126) that conantokin-G enhanced strychnine-insensitive [^{3}H]-glycine binding to rat forebrain membranes in a concentration-dependent manner (ED$_{50}$ 152 nM). Neither competitive nor noncompetitive NMDA antagonists mimic this effect on [^{3}H]glycine binding.

Two studies suggested that conantokin-G is a highly subtype-specific NMDA receptor antagonist. At concentrations up to 10 μM, conantokin-G had minimal if any effects on NR1a/NR2a, NR1a/NR2C, and NR1a/NR2D subunit combinations expressed heterologously. However, conantokin-G blocked Nr1a/NR2B heteromeric receptors. The block did not show either use dependence or voltage dependence. Furthermore, coapplication of the competitive antagonist (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-

phosphonic acid (CPP) prevented development of block by conantokin-G, consistent with competitive antagonism at the NR1a/NR2B subtype (38). With the use of HEK 293 cells expressing cloned rat NMDA receptors, similar results were found by Klein et al. (93); conantokin-G blocked the NR1a/NR2B receptor but failed to block the NR1a/NR2a subunit combination, even at a concentration of 50 μM. However, this strong selectivity for the NR2B subunit was not found by Wittekindt et al. (192). The discrepancy regarding the subtype selectivity of conantokin-G needs to be resolved.

Other conantokin peptides (see Table 4) have been characterized from a variety of fish-hunting *Conus*: conantokin-T (61), conantokin-R (187, 188), and conantokin-L (83). In contrast to conantokin-G, these peptides do not appear to discriminate between NR2B- and NR2a-containing complexes (92, 188); however, conantokin-R still exhibited NMDA receptor subtype selectivity since the peptide did not inhibit NR2D-containing receptor complexes (188). The structure of conantokin-T has been determined by several groups (104, 171, 185).

The discovery of conantokin-R (188) was notable for the demonstration that this peptide was not only an antagonist of NMDA receptors, but had potent anticonvul-

sant activity accompanied by relatively low behavioral toxicity using the Frings audiogenic seizure mouse as an animal model (188). Curiously, although conantokin-L has a sequence that is almost identical to that of conantokin-R except at the COOH-terminal end, and although it is as potent as conantokin-R as an NMDA receptor antagonist in vitro, it has a much reduced anticonvulsant potency compared with conantokin-R in the Frings mouse model (83). It is notable that conantokin-G is also a potent anticonvulsant and has been through human clinical trials for this therapeutic application; in addition, it has been demonstrated to be neuroprotective in rat models of ischemia (190, 191).

IV. OVERVIEW OF *CONUS* VENOM COMPONENTS: PERSPECTIVES

A. Other Venom Components

In sections II and III, we focused on *Conus* peptides that target either ligand-gated or voltage-gated ion channels in mammalian systems. We will briefly summarize work on the other *Conus* venom components that do not

TABLE 5. *Miscellaneous* Conus *venom components*

Peptide	*Conus* Species	Target/Function	Sequence or Amino Acid Number	Reference Nos.
GPCR targeting				
Conopressin-G	*C. geographus*	Vasopressin receptor	CFIRNCPLG*	31
Contulakin-G	*C. geographus*	Neurotensin receptor (?)	ZSEEGGSNAT†KKPYIL	29
ρ-Conotoxin TIA	*C. tulipa*	α_1-Adrenergic receptor	FNWRCCLIPACRRNHKKFC*	164
Other targets or molecular target not established				
mr5a; χ-conotoxin MrIA	*C. marmoreus*	Norepinephrine transporter	NGVCCGYKLCHOC	118, 164
Spasmodic peptide (spasmodic-Tx)	*C. textile*	(Target unknown)	GCNNSCQγHSDCγSHCICTFRGCGAVN*	106
"μ-PnIVA"	*C. pennaceus*	Molluscan Na channels	CCKYGWTCLLGCSPCGC	52
γ-Conotoxin PnVIIA	*C. pennaceus*	(Molluscan pacemaker channels?)	DCTSWFGRCTVNSγCCSNSCDQTYCγLYAFOS	53
tx5a; TxIX	*C. textile*	(Reduces Ca^{2+} flux)	γCCγDGWCCT†AAO	155, 184
Conorfamide	*C. spurius*	(RFamide receptors; ENaC channels?)	GPMGWVPVFYRF*	113
Contryphan-R	*C. radiatus*	(Modulation of Ca^{2+}-activated K channels?)	GCOWEPWC*	84, 115
"Bromosleeper" peptide	*C. radiatus*	(Target unknown)	WATIDγCγγTCNVTFKTCCGOOGDWQCVγACPV	28
Small molecules				
Serotonin	*C. imperialis*	(Neurotransmitter)		48
Arachidonic acid	*C. textile*	(Effector; precursor for prostaglandins, leukotrienes		133
Large polypeptides				
Conodipine	*C. magus*	Phospholipase A_2	137	120
γ-Glutamyl carboxylase	*C. textile*	Posttranslational modification of Glu	811	9
Conophysin	*C. radiatus*	Carrier of conopressin (?)	84	105
Propeller activity	*C. striatus*	(?)	86	46

γ, γ-Carboxyglutamate; T†, *O*-glycosylated threonine; W, D-tryptophan; O, hydroxyproline. * COOH-terminal amidation.

fall into those categories (see Table 5). Several conopeptide families have been characterized that target ion channels but were not discussed above because a definite molecular target had not been identified. Among the conopeptides reported to have an ion channel target are γ-conotoxin PnVIIA from *Conus pennaceus* (53) and peptide μ-PnIVA from *C. pennaceus* (52); these target molluscan pacemaker channels and voltage-gated Na channels, respectively (the peptide designated M-PnIVA is not a conventional member of the μ-conotoxin family as presently defined). In addition, conorfamide from *Conus spurius* venom may be an agonist for neuropeptide FMRFamide-gated ion channels, as well as a potent modulator of epithelial Na channels in mammalian systems (113). Finally, evidence has recently been presented that a member of the contryphan family, contryphan-Vn (116), modulates the activity of Ca-activated K channels (115). It has not been determined whether or not this is a direct effect on the channel. Thus the spectrum of conopeptide families that target ion channels is likely to be significantly greater than indicated in Tables 1–4.

Additionally, a number of conopeptides have been shown to target G protein-coupled receptors. Interestingly, two of these are agonists: conopressin-G is an agonist for the vasopressin receptor (31), and contulakin-G is believed to be a subtype-specific agonist of a receptor for the neurotensin peptide family (29). Only one peptide so far, ρ-conotoxin TIA, which has been shown to target the α_1-adrenergic receptor, is a G protein-coupled receptor antagonist (164). For some peptides, such as peptide tx5a (also called TxIX), the target is likely either an ion channel or a G protein-coupled receptor (155, 184). Evidence that this peptide reduces Ca flux in presynaptic termini has been presented, but the actual molecular target (which may be a Ca channel or a G protein-coupled receptor that affects Ca channels) has not been definitively identified. Finally, a number of peptides that are demonstrably biologically active which have been biochemically characterized have either different or unknown targeting specificity; examples include χ-conotoxin MrIA, also called mr5a, a member of the T-superfamily (118) that has been reported to target the norepinephrine transporter (164), and the spasmodic peptides and the contryphans, whose targets have not been defined (75).

In addition to peptides, a variety of small molecules have been detected in *Conus* venoms. Only two of these have been identified, serotonin from the venom of *C. imperialis* (48) and arachidonic acid (133), which is present at high levels in the venom of *Conus textile*. It is notable that in the case of serotonin, all other *Conus* venoms examined except *C. imperialis* did not have this bioactive amine present at detectable levels.

Finally, larger polypeptides have been described from *Conus* venom ducts. Some of these are probably important for paralysis, such as the propeller activity from *C. striatus* (46), which is 86 amino acids in length and is a highly potent component of this venom; this peptide causes potent excitotoxic effects in both fish and mice. Some of the larger venom polypeptides may have carrier functions, such as conophysin from the venom of *C. radiatus* (105), which may play this role for conopressin. Other polypeptides detected may be posttranslational modification enzymes, such as the γ-glutamyl carboxylase from *C. textile*. There may be a variety of components of *Conus* venoms that are enzymes which play a role in envenomation; the best-characterized is conodipine from *Conus magus*, a novel phospholipase A_2 (120).

B. Perspectives

Although this review has focused on conopeptides targeted to ion channels, the immediately preceding section gives a more balanced picture of the overall complexity of *Conus* venoms. Clearly, these venoms are biochemically much more diverse than might be gleaned from the conopeptide families whose mechanisms have been elucidated; a largely undefined complement of small molecules and larger polypeptides are also present. Furthermore, the physiological targets of venom components are clearly more diverse than the classes of ion channels described in sections II and III.

However, much of this additional, largely undefined diversity can be accommodated in the general framework of cone snails being specialists in neuropharmacology, using combination drug therapy as a prominent feature of the venom strategy for achieving the desired biological end points beneficial to the cone snail. We have described how peptides acting together on discrete ion channel targets can function as a coordinated "cabal." It is reasonable to expect that most of the small molecules as well as the larger polypeptides in the venom could function similarly as cabal components. It is noteworthy in this respect that one of the small molecules that has been identified in a particular venom, arachidonic acid from *C. textile*, would be the first product of one of the enzymes identified from a different venom, conodipine-M, which has phospholipase A_2 activity that would be expected to directly produce arachidonic acid. Thus venom constituents that are biochemically unrelated may in fact have convergent physiological roles. In many cases, it is difficult to discern what these roles may be from the individual components, since it is the synergy between multiple venom components that probably makes *Conus* venoms particularly effective neuropharmacological brews.

The study of *Conus* venoms may have several longer term impacts on human pharmacology. Individual *Conus* venom components have been shown to have therapeutic potential, and several have reached the stage of human

clinical trials. The development of two ω-conotoxins, ω-conotoxin MVIIA (generic name ziconotide, commercial name Prialt from Elan Pharmaceuticals) and ω-conotoxin CVID as drugs for intractable pain (103, 127, 141, 138) was described in section IID. Conantokin-G (that targets NMDA receptors; see sect. IIIE) and contulakin-G, likely a neurotensin receptor agonist (29), are being developed for epilepsy and pain, respectively; both have been through phase I human clinical trials. Another peptide, the nicotinic receptor antagonist vg1 (see Table 3), is being explored as a therapeutic agent for pain. The potential of several conopeptides as possible neuroprotective agents during ischemic episodes has also been investigated (190, 191). Thus a number of diverse *Conus* venom constituents have shown therapeutic promise, most likely arising from their unusually narrow targeting specificity.

However, it is unlikely these venom components that are being developed for human therapeutic use are used endogenously as individual pharmaceutical agents, independent of other venom constituents. As we described above, venoms are generally organized into cabals of toxins that act together. Thus one message from the analysis of cone snail venoms is that combination drug therapy may make sense for a number of human therapeutic situations. It has been recent practice to discourage the use of pharmacological mixtures for therapeutic use; only when the need has become extremely desperate, as was the case during the acquired immunodeficiency syndrome crisis and for the more intractable cancers, has combination drug therapy been explored at all. However, the cabals in cone snail venoms indicate that using combinations of pharmacological agents can be extremely effective; these may also be a guide as to which potential therapeutic targets are reasonable to try to use in the design of drug combinations for some of the more intractable pathological states. An important sidebar to this is that combination drug therapy will not be optimally effective until the components of the combination are highly specifically targeted; otherwise, side effect profiles will probably not be tolerable. In this respect, the cone snails are clearly more experienced and sophisticated than are the major pharmaceutical companies.

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