CONUS PEPTIDES AS TOOLS FOR THE NEUROSCIENTIST

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Recombinant DNA technology has had a powerful impact on understanding receptors and ion channels, the key components in the nervous system that are involved in intercellular communication. Cloning genes encoding these proteins has revealed that for every receptor and ion channel type, multiple molecular forms exist. In a sense, the effect of molecular biology on neuroscience has been to induce a significant shift in philosophical orientation: the elegant analyses of Hodgkin and Huxley had traditionally led to a focus on general principles, i.e., action potential generation by the "universal" sodium and potassium channel. The reality that there are many dozens of different potassium channels provides a new challenge: to understand the specialized physiological roles of different receptor and ion channel subtypes present in the nervous system.

One effective approach for investigating the physiological function of particular receptor or ion channel subtypes is to have agents which discriminate between different subtypes. Thus, an understanding of the role of different types of potassium channels would be significantly facilitated by ligands that were highly specific for a given potassium channel subtype. There is one natural ligand system that has considerable potential for investigating the physiological functions of different receptor subtypes. These are the *Conus* peptides, found in the venoms of the predatory gastropod snails belonging to the genus *Conus* (Olivera et al., 1990).

Biological background

There are approximately 500 different species of cone snails (Walls, 1979); these occur in all tropical marine waters (see Frontispiece). The richest con-

centration occurs in the Indo-Pacific, where over 25 different species of *Conus* might inhabit a single reef (Kohn and Nybakken, 1975). The cone snails are among the most abundant predatory animals of tropical marine communities.

The venoms of cone snails are extraordinarily complex, and particularly rich in small peptides between 10-30 amino acids in length (Olivera et al., 1985b; Olivera et al; 1990). The peptides comprise a remarkable pharmacological spectrum, and a complete catalog of all biologically active peptides in even one *Conus* venom has not yet been achieved. It appears that each venom may have from 50-200 different peptides, each targeted to a particular receptor or ion channel subtype.

However, the few comparative studies between venoms from different species in the genus have revealed a remarkable divergence of peptide sequences. Indeed, it appears that part of the reason for the success of the genus *Conus* is the rapid evolution of venom peptide sequences due to specialized genetic mechanisms which cause hypermutation (Olivera et al., 1990; Woodward et al., 1990). Thus, it is not atypical to find two homologous peptides with the same physiological effects from different species of *Conus* with only the arrangement of the cysteine residues conserved but 50% or more of the non-cysteine amino acids non-identical (see Table I). The hypermutation apparently occurs as species diverge; constancy of peptide sequence between individuals within a single species has been found. The large number of cone snail species means that a large homologous series of peptides which differ significantly in sequence from each other are available for studying a particular receptor or ion channel subtype.

Conus peptides as subtype-discriminating ligands

Several properties of the *Conus* peptide system make these agents particularly promising to explore as ligands that discriminate between receptorsubtypes. Venomous animals which inject their venom into prey are under strong selection for efficient use of components in the venom. For the relatively slow-moving cone snails that generally go after more rapidly-moving prey, the speed of paralysis of the prey is a major consideration.

These biological factors may lead to selection for very highly specific ligands that discriminate between closely related subtypes.

Species	Peptide	Sequence
ω-conotoxins (Target: voltage-sensitive Ca channels)		
C. geographus	GVIA	CKSOGSSCSOTSYNCCRSCNOYTKRCY*
C. striatus	SVIA	CRSSGSOCGVTSICCGRCYRGKCT*
C. magus	MVIIC	CKGKGAPCRKTMYDCCSGSCGRRGKC*
α-conotoxins (Target: nicotinic acetylcholine receptors)		
C. geographus	GI	ECCNPACGRHYSC*
C. striatus	SI	ICCNPACGPKYSC*
C. magus	MI	GRCCHPACGKNYSC*

Table I. Conotoxin Sequences: Six of the approximately 100 Conus peptide sequences that have been determined to date. One ω-conotoxin and α-conotoxin is shown from three Conus species. Note how the arrangement of cysteine residues is conserved within the ω- and α-conotoxin families, but is different between the two groups. The references for the ω-conotoxins are: GVIA (Olivera et al., 1984); SVIA (Ramilo et al, 1992); MVIIC (Hillyard et al., 1992). For the α-conotoxins, the references are: GI (Gray et al., 1981); MI (McIntosh et al., 1982); SI (Zafaralla et al., 1988). Except for O (hydroxyproline), standard one-letter abbreviations for amino acids are used.

A specific example are the calcium channel-targeted toxins that are found in fish-hunting *Conus* venoms, the ω-conotoxins (Olivera et al., 1991). The available molecular genetic data suggests that different calcium channel complexes are closely related to each other. Nevertheless, all ω-conotoxins found in fish-hunting cone snails appear to be rather specific for neuronal high-threshold voltage-sensitive calcium channels.

One possible reason for Ca-channel subtype selectivity is that before an ω-conotoxin can reach its physiologically relevant target (i.e., voltage-sensitive calcium channels at motor nerve endings which control the release of acetyl-

choline), the peptide must first enter the vasculature and may therefore be exposed to other subtypes of calcium channels, such as Ca channels in smooth muscle and in skeletal muscle. If the toxin had any significant affinity for these related voltage-sensitive calcium channels, it would result in the toxin being significantly delayed in its progression towards the physiologically relevant target. The venomous predator would have to overproduce the toxin so that a sufficient amount could get to neuromuscular junctions rapidly. What appears to have evolved are peptides that can discriminate by many orders of magnitude between neuronal and non-neuronal calcium channels. Thus, *Conus* peptides may possess structural features that allow them to strongly discriminate between closely-related target subtypes.

In addition to these biological considerations, another relevant factor is the genetic and evolutionary strategy of the genus. As discussed above, evidence has accumulated which suggests that the snails rapidly evolve new peptides by a novel mechanism. In many ways, the *Conus* peptide system has analogies to the generation of antibody diversity by the mammalian immune system. The cone snails have venom peptides which exhibit relative structural conservatism (i.e., the disulfide bridging patterns which are found in venom peptides across the whole genus) (Hillyard et al., 1989). However, ligand diversity is generated by mechanisms that very rapidly change amino acid sequences in the genes encoding the *Conus* peptides. Thus, when various ω-conotoxin sequences are compared, there are clearly constant and hypervariable regions; within the mature peptide, the constant amino acids comprise primarily the cysteine framework responsible for disulfide crosslinks (see Table I).

Strong selection for subtype specific ligands combined with hypermutation of peptide sequences results in sets of subtype selective, high affinity peptide ligands for particular receptors. Thus, the Ca channel-specific peptides, the ω-conotoxins, can presumably cause inhibition of the presynaptic calcium channels in teleost fish, the prey of the piscivorous cone snails. However, a large number of sequence solutions which can differ by as much as 70% in the non-cysteine amino acids have been identified (see Table I). Such peptides constitute a powerful set of ligands for examining the many molecular forms of voltage-sensitive calcium channels in the mammalian brain.

Overlapping subtype specificity of *Conus* peptides.

The generation of many peptides targeting to the same general site of a receptor or ion channel may seem like useless redundancy from the neuroscientist's point-of-view. We will argue the contrary: given the very large number of closely related subtypes in the mammalian central nervous system, sets of ligands with overlapping specificity should be useful for defining receptor-subtype function. Among *Conus* peptides, the calcium channel-targeted peptides, the ω-conotoxins, have provided the most extensive data set to date. It is useful in this context to compare two specific peptides, ω-conotoxin GVIA (Olivera et al., 1984) from *Conus geographus* venom and ω-conotoxin MVIIC (Hillyard et al., 1992) from *Conus magus* venom (the sequences of these peptides are shown in Table I).

ω-Conotoxin GVIA has become one of the most widely used of all toxin ligands in neuroscience. It is the standard tool for pharmacologically defining the N-type voltage-sensitive calcium channel in mammalian systems (Olivera et al; 1984; Nowycky et al., 1985; Bean, 1989; Yoshikami et al., 1989; Tsien et al., 1991). (It is likely that what is pharmacologically defined as the N-subtype is not a homogeneous molecular set of voltage-sensitive calcium channels, and may very well comprise numerous molecular species. Definitive answers should be provided by cloning efforts underway in several laboratories).

If certain standard assays were used, ω-conotoxin MVIIC from *Conus magus* might seem to be a lower affinity sequence variant of ω-conotoxin GVIA, except that it is from a different cone snail. Like ω-conotoxin GVIA, MVIIC paralyzes fish. If unlabeled MVIIC is used to compete for binding with labeled ω-conotoxin GVIA, it displaces all of the radiolabeled ω-conotoxin GVIA indicating that the MVIIC peptide competes for the GVIA binding sites. However, a very simple and effective test of whether these homologous peptides have the same subtype specificity in the mammalian central nervous system is the direct injection of such peptides into the CNS. It became immediately obvious from this test that the two peptides must have significantly different specificity (Hillyard et al., 1992).

When ω-conotoxin GVIA is injected directly into the central nervous system of mice, the animals exhibit a characteristic "shaking syndrome" (Olivera et al., 1984). In mice, injection of large amounts of GVIA peptide does not cause lethality, but merely intensifies the characteristic shaking. In contrast, injection of ω-conotoxin MVIIC results in a dramatically different symptomatology: instead of shaking, the mice arch their backs, gasp for breath and death results in a few minutes at relatively low doses (Hillyard et al., 1992). Though a crude test, the very different *in vivo* symptomatologies clearly indicate that the two peptides are not identical in their receptor target subtype-specificity.

A variety of evidence has now established that ω-conotoxin MVIIC is a broader spectrum ligand in the mammalian central nervous system than is ω-conotoxin GVIA (Hillyard et al., 1992). Although the MVIIC peptide will bind all of the GVIA sites, the converse is not true under physiological conditions. Thus, there are Ca channels that are sensitive to MVIIC but insensitive to GVIA. This differential sensitivity provides an operational definition of new Ca channel subtypes: 1) an MVIIC-sensitive, GVIA-insensitive class, and 2) the N-type Ca channel which is MVIIC and GVIA sensitive. By characterizing more calcium channel-targeted ligands, each with a different pharmacological spectrum of subtype-specificity, it should become possible to define progressively more new classes of voltage-sensitive calcium channels.

An additional strategy for dissecting receptor and ion channel subtypes is to obtain not just one set of ligands with overlapping subtype specificity (such as the ω-conotoxins from piscivorous cone snails) but to cross-compare one set of ligands with an independent set that has specificity centered on different subtypes. For voltage-sensitive calcium channels, there are several alternatives for a second ligand set. One possibility are cone snails that are not piscivorous, which may have developed calcium channel ligands that have different subtype-specificity in the mammalian central nervous system. Many *Conus* species do not eat fish, but eat other animals such as polychaete worms or molluscs (Kohn, 1959). The possibility of using these venoms for isolating novel sets of calcium channel-targeted peptides that have a different specificity is presently being explored.

Another strategy is to use an entirely different, taxonomically distant set of venomous animals. The best present candidate are the spiders, which also use a strategy of paralysis that involves inhibition of presynaptic voltage-sensitive calcium channels. Cross-comparison of the set of calcium channel-targeted toxins in one spider venom (the ω-agatoxins) to those of fish-hunting cone snails (the ω-conotoxins) has proven to be extraordinarily useful in distinguishing various channel subtypes (Olivera et al., 1991). It is anticipated that multiple overlapping sets of such toxins will be increasingly common tools for the neurobiologist in the years to come.

Functional roles of receptor subtypes: developmental and phylogenetic considerations.

Another general question regarding receptor subtypes is whether a specific receptor subtype plays different functions either in different stages of a given organism's development, or between different organisms. Such comparative questions can also be approached by using receptor subtype-specific ligands to determine how a specific subtype changes as a function of development, or during evolution.

In the *Conus* peptide system, there is already clear evidence for interesting developmental specificity with respect to the *in vivo* effects of some *Conus* peptides. For example, in mice, ω-conotoxin GVIA is not lethal to adult animals, but neonatal animals are killed by this peptide (Myers et al., 1990). The phenotype seen is a progressively increasing difficulty in breathing. Whether this is because there are ω-conotoxin GVIA-sensitive subtypes in respiratory circuits during the neonatal period, or whether there are functional bypasses of ω-conotoxin GVIA-sensitive channels later in development remains to be established.

A similar striking change in phenotype is seen for a different *Conus* peptide, the NMDA-receptor specific peptide conantokin-G (McIntosh et al., 1984; Mena et al., 1990; Hammerland et al., 1992). The conantokins elicit a sleep-like state in younger animals, under two weeks of age (Olivera et al., 1985a). In contrast, when the peptide is injected into older mice (> 3 weeks of age), instead of sleep, a distinctive hyperactivity is observed where the mice are constantly climbing the sides of their cages and running from corner to cor-

ner (Rivier et al., 1987). Presumably, the strikingly different behavior between two and three weeks may either be a consequence of a new subtype of NMDA receptor becoming dominant during the adult period, or rewiring of the circuitry so that the same receptors are affected, but different behavioral effects ensue. It remains to be established which of these possibilities is in fact correct. However, in both of these cases the peptides provide an unusual opportunity to examine the functional biology of specific receptor subtypes as a function of development.

There are clearly striking differences in phylogenetic specificity as well. Thus, some of the α-conotoxins which are effective on teleost fish have proven to be relatively inactive in mammalian systems; in contrast, some homologs seem almost as active in both teleosts and mammals. Examples are α-conotoxin GI from *Conus geographus* venom vs. α-conotoxin SI from *Conus striatus* venom (Zafaralla et al., 1988). The former is a potent paralytic toxin in mice, while the latter is inactive. Because these peptides are quite closely homologous, it becomes possible to use the peptides to investigate whether or not specific amino acid changes in either the ligand or the receptor are responsible for the different biological effects of the homologous peptides. In addition, because the 2D NMR structure of these peptides has been determined (Pardi et al., 1989; Kobayashi et al., 1989; Christensen et al., 1992), the *Conus* peptides also provide a defined three-dimensional framework for investigating potentially important phylogenetic changes in receptor structure.

Perspectives: *Conus* peptides and the study of the multiplicity of receptor subtypes

The challenge of obtaining subtype-specific ligands is enormously important for progress in molecular neuroscience, but is clearly a massive undertaking. Given the accelerating number of subtypes for every receptor and ion channel type being elucidated by molecular cloning, the number of molecular forms of receptors and ion channels seems to increase much faster than the discovery of subtype-specific ligands. However, to a large extent, this is because the necessary technology to be able to address how to obtain a subtype-specific ligand for a particular molecular species of receptor or ion channel has not yet been developed.

The *Conus* peptide system is promising not only because *Conus* peptides have intrinsic subtype specificity, and because there are clearly a wide variety of natural peptide ligands in *Conus* venoms, but also because the generation of ligands that are highly subtype-specific can potentially be organized into a systematic technology based on the natural *Conus* system. The peptides that are present in *Conus* venoms have an advantage over small organic ligands in that they are the direct products of genes. Unlike many pharmacologically active natural products, which must be produced by enzymatic synthesis, *Conus* peptides are directly encoded by messenger RNA's and their primary structures can readily be determined by nucleic acid sequencing methods. However, in contrast to most of the polypeptide toxins that have been evolved by venomous animals (i.e., snakes, spiders and scorpions, for example), the *Conus* peptides are significantly smaller, being in a size range that is amenable to present methodologies for chemical synthesis. Consequently, an analysis of their tertiary structure is, at least in principle, more accessible.

Most exciting however, is the possibility that once natural "lead peptides" have been obtained, that these peptides can be quickly refined so that specific properties can be selected that may not be found in the complement of natural *Conus* peptides (Olivera et al., 1992). Thus, because *Conus* peptides are the direct translation products of genes, the great progress in gene technology can be applied to obtain a mutagenized *Conus* peptide with properties that may be much more desirable for the neuroscientist to use. Thus, the future of *Conus* peptides in neuroscience really has two components. On the one hand, the discovery of more novel natural *Conus* peptides which will become standard tools for the neuroscientist to investigate physiological processes (such as ω-conotoxin GVIA) should continue at an accelerating pace. However (and more significant), around this natural peptide system, a new technology can be developed which will permit the generation of *Conus* peptide molecules which were never produced by the snails in their venom ducts, but which may serve as "magic bullets" that the neuroscientist can use in understanding how the nervous system functions.

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