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Conotoxins and Their Potential Pharmaceutical Applications

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Abstract

The neurotoxins isolated from cone shell venoms are a diverse group of small, disulfiderich peptides. Most of the active peptides isolated to date have been shown to specifically target various components of neural transmission, and have generally demonstrated high specificities for ion channel and receptor types and subtypes. The specificity of conotoxins is one of the attributes that make them valuable diagnostic tools in the characterisation of neural pathways, as therapeutic agents in medicine, and potentially as biodegradable toxic agents in agroveterinary applications. The number of novel, active peptides within the numerous *Conus* species is considered to be enormous. Currently, however, relatively few peptides have been characterised. In this article, we review current research on conotoxins with a focus on drug potential being developed at the University of Queensland, Australia.

Key words: ion channels; sodium channel; acetylcholine; nicotinic receptor; synaptic transmission; peptide; gene cloning; NMR spectroscopy; crystal structure

Introduction

Ion channels as drug targets

Voltage-dependent ion channels are intrinsic membrane proteins that play an important role in fast communication in excitable cells. A short stretch of amino acids, the pore region, is the sole determinant of cation selectivity and also forms the binding site for many channel blockers. Toxins that interact intimately with this region can be used as structural templates to deduce the spatial organisation of the pore region of the ion channels. These models of pore structure are valuable for understanding the mechanisms of ion permeation, and ultimately may be useful for the rational design of drugs that modify the function of ion channels in clinical conditions such as stroke, pain, or epilepsy.

Broadly, ion channels have structural and functional similarities, but even within a class of ion channels there are significant differences that can be targeted in drug applications. The diversity and distribution of ion channel types and subtypes being uncovered through the use of molecular biology and toxin probes present an exciting opportunity for the discovery of new therapeutics which are specific for channel subtypes involved in disease states. The various ion channels to be considered will be examined briefly in turn.

Nicotinic Acetylcholine Receptor-Channels

The nicotinic acetylcholine receptor (nAChR) is part of the ligand-gated ion channel superfamily, which includes the GABAA, serotonin, and glutamate (NMDA, AMPA, kainate) receptors. All ligand-gated ion channels are large, membrane-bound pentamers with various subunit compositions. These receptors have several conserved features. Ligand-gated ion channels are pentamers, with each subunit containing four transmembrane helices (M1 to M4), with the M2 helix lining the ion channel lumen and providing it selectivity. Binding of an endogenous ligand to a large, extracellular domain remote to the M2 helix brings about a conformational change in the M2 helices that

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causes the pore to open. Due to the size of these receptors (~290 kDa), the only direct structure determinations have been of low resolution (~9 Å) using electron microscopy [Unwin, 1998].

Nicotinic ACh receptors are found throughout the central and peripheral nervous systems, with distinct genes encoding the nAChR subunits which form a heteropentameric ion channel complex selective for cations. The muscle-subtype nAChR has been well characterised due to the availability of specific probes (e.g., a-bungarotoxin) and has the subunit composition (a1)2b1dg or e in mature muscle. In mammalian central and autonomic neurones and adrenal medulla, the neuronal nAChRs are composed of a and b subunits only. At least seven different a subunits (a2–a7 and a9) and three b subunits (b2–b4) have been identified and it has been shown that a2, a3, and a4 can combine with b2 or b4 to form functional channels in the *Xenopus* oocyte expression system [McGehee and Role, 1995]. In addition, a7 and a9 subunits can be expressed as functional homooligomers in this system, with the a7 gene product being a-bungarotoxin-sensitive and highly permeable to Ca2+ [Colquhoun and Patrick, 1997]. Although these neuronal nAChR subunits are homologous with one another, each functional subunit combination is physiologically and pharmacologically distinct. This may account for the diversity of neuronal nAChRs observed in vivo. For example, the a5 subunit appears to participate in nAChRs expressed in heterologous systems and primary neurones and contributes to the pore lining of functionally unique nAChRs. Recent studies using single cell RT-PCR analysis of nAChR gene transcripts indicate that multiple nAChR subtypes are expressed by individual rat intracardiac neurones and that the combination of subtypes expressed varies among cells [Poth et al., 1997]. The development of specific pharmacological probes for neuronal nAChR subunits will provide new insight into the structural composition and functional role of the different neuronal nAChRs subtypes.

Activation of distinct subtypes of these presynaptic nAChRs by nicotinic agonists can selectively regulate the release of different neurotransmitters, including dopamine, norepinephrine, glutamate, and acetylcholine [Kulak et al., 1997; Kaiser et al., 1998; Picciotto et al., 1998]. Such receptors have also been implicated in the pathophysiology of several neuropsychiatric disorders, including schizophrenia, Alzheimer's disease, Parkinson's disease, and Tourette's syndrome [Kulak et al., 1997]. Despite their importance, few of the nicotinic receptor antagonists identified to date are highly selective between the multiple neuronal nAChR subtypes. Thus, the ability of recently discovered a-conotoxins — small (12–19 amino acids), rigid, highly disulfide-bonded peptides isolated from marine snails of the genus *Conus* — to target neuronal nAChR subunits with high specificity has considerable significance for both basic neuroscience and potential drug development.

Sodium Channels

Sodium channels consist of three separate and biochemically separable protein subunits, the a plus b-1 and b-2 auxiliary subunits, which comprise the channel in a 1:1:1 stoichiometry. The a-subunit is a transmembrane glycoprotein of approximately 260 kDa molecular weight that binds a diverse range of neurotoxins at specific positions on its surface (six or seven sites are currently identified). The two b-subunits have smaller molecular weights (~30 kDa each) and are integral membrane glycoproteins. Numerous models of sodium channel a-subunit structure have appeared, based on primary sequence data [Noda et al., 1984]. All show four highly homologous regions of sequence domains, labeled I–IV, with each domain containing six transmembrane helices, denoted S1–S6. The S5 and S6 segments of each domain are highly nonpolar, the S1, S2, and S3 segments are relatively nonpolar, with just a few charged sidechains, but the S4 segments within each domain have the distinctive feature that every third residue is positively charged (mostly arginines). The S4 segments are believed to move upward on depolarisation to open the activation gate (m gate) and allow the selective influx of sodium ions. In the process, voltage-dependent movement of an IFM particle to interact with adjacent intracellular loops is facilitated and inactivation occurs, blocking the further flow of ions (Fig. 1).

There is considerable structural homology among the three types of brain Na+ channel a-subunits (I, II, and III), the m1-sodium channel a-subunit from adult skeletal muscle, and the h1 sodium channel a-subunit from heart and denervated muscle. Despite these similarities, considerable pharmacological diversity exists. For example, tetrodotoxin (TTX) blocks the brain types I, II, and III at nanomolar concentrations, and the h1 form from the heart at micromolar concentrations.

Until recently, there was no hard evidence to indicate that pharmacologically distinct forms of neuronal sodium channels are expressed in sensory neurons, and thus no evidence that a specific Na+ channel pathway could be modulated to control particular diseases. The newly discovered TTX-insensitive sodium channel, named PN3 or SNS [Sangameswaran et al., 1996], which is located specifically in sensory neurons, represents one of a number of potential Na+ channel targets for drug discovery. Additional neuronal pathways for therapeutic intervention may also be uncovered using conopeptides such as m-conotoxin PIIIA, the first conopeptide to distinguish amongst neuronal TTX-sensitive Na+ channels [Shon et al., 1998; Watson et al., 1998].

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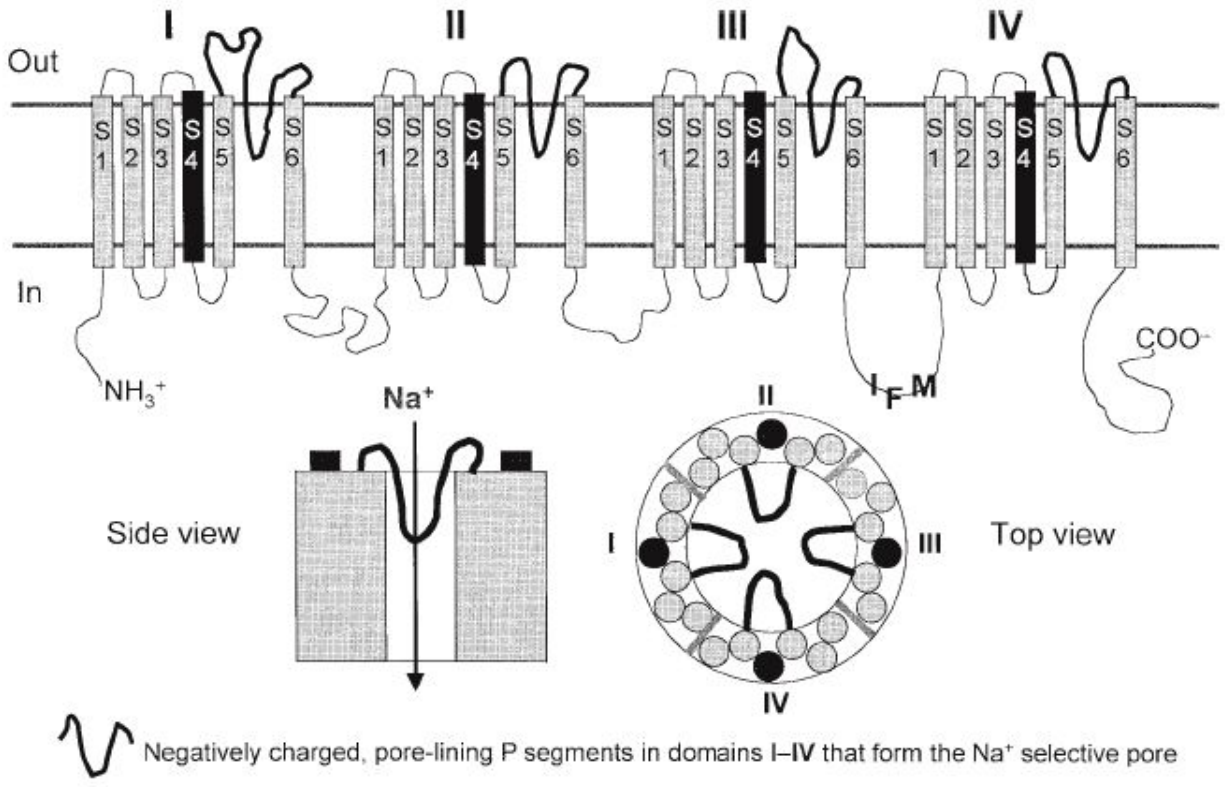

Fig. 1. Transmembrane segments of the sodium channel and a model of their construction into the α-subunit that forms the pore of the channel. The repeating nature of the transmembrane domains is emphasised, as is the IFM particle that acts as the inactivation gate. The nature of the residues comprising the activation gate is presently not known. Interestingly, the calcium channel has the same general structure.

Calcium Channels

Structurally, the calcium channels are closely related to sodium channels, with the main difference being the positioning and nature of the residues that line the selectivity filter in the pore of the channel. There are at least six pharmacologically distinct calcium channels types, including L-, N-, P/Q-, T, and R-type calcium channels, and within each group are multiple subtypes that are presently less easy to distinguish. In the nervous system, several types of ion channels may contribute to processes such as neurotransmitter release, with the ratio and role for each type varying among different nervous tissues [Olivera et al., 1994]. This situation provides the possibility for selective modulation of nerve function with type and subtype selective modulators that may allow the selective treatment of conditions such as pain and stroke. The w-conotoxins have been of enormous importance as physiological tools, with currently one peptide (MVIIA or Ziconitide) in clinical trials for pain and stroke.

Potassium Channels

There are numerous types of potassium channel, each with its own distinctive electrophysiological and pharmacological properties; what they all have in common is that they tend to stabilise the membrane potential at the K+ equilibrium potential. DNA sequencing reveals that the potassium channels encoded by *Drosophila* and vertebrate genes all resemble a single domain of the voltage-dependent sodium channel [Jan and Jan, 1997]. Voltage-dependent potassium channels are tetrameric homo-oligomers organised in axial fourfold symmetry around the K+-selective pore. Analogous to voltage-dependent sodium and calcium channels, the S4 transmembrane segment carries a cluster of positively charged residues and is thought to act as the voltage sen- sor for channel activation. Site-directed mutagenesis studies, coupled with the use of selective toxins, have proved invaluable in unraveling which residues of the potassium channel protein are functionally important. Recently, the crystal structure of a K+ channel has been determined [Doyle et al., 1998]. The pore structure determined previously from

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toxin binding interaction studies has proved to be remarkably predictive [Miller, 1995], though it lacks the structural detail obtained by X-ray crystallography. k-Conotoxin PVIIA is a new structural class of K+ channel blocking peptide that binds in a voltage- sensitive manner to the outer vestibule of the channel [Scanlon et al., 1997].

Discovery and Characterisation of Novel Conotoxins

Tropical waters, especially in coral reef ecosystems, house an extraordinary diversity of invertebrate species, many of whom use novel bioactive compounds as part of defensive or prey capture strategies. The cone shells which comprise a group of some 500+ predatory molluscs are the most specialised, with venoms that target fish, worms, and other molluscs. The venom is injected through a harpoon-like apparatus and contains a complex mix of small, constrained peptides which contain 10–40 amino acids and up to five disulfide bonds [Myers et al., 1993]. This cocktail of peptides targets a diverse range of voltage-sensitive sodium, calcium, and potassium channels and *N*-methyl-d-aspartate, glutamate, vasopressin, serotonin, and acetylcholine receptors, which leads to an immediate and efficient immobilisation of the prey. The conotoxins present in the venom have been divided into a number of major classes based on their pharmacological activity and cysteine frameworks (Table 1). Their high potency and specificity, and convenient chemical synthesis, also make the conotoxins attractive leads in drug design programs. In addition to the conotoxins being among the smallest bioactive peptides, they are unusual in containing a high density of cysteine residues and posttranslation modifications, including hydroxylation, carboxylation, amidation, sulphation, and bromination. These features often complicate their chemical characterisation and occasionally their chemical synthesis. All major classes of conotoxins have been identified through initial in vitro or in vivo functional assays [Olivera et al., 1990]. Screening based on receptor-binding displacement of radiolabeled ligands is also playing a major role. At the 3D Centre, University of Queensland, sensitive 125I-GVIA and 125I-MVIIC assays have been established for rat and human brain preparations to allow for the isolation of new w-conotoxins. More recently, other chemical and molecular biology approaches have facilitated the identification and primary structure determination of new conotoxins. In practice, all of these approaches are used in concert to discover new conotoxins.

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TABLE 1. Six Major Classes of Conopeptides and Their Disulfide Connectivity

α-conopeptides (2 loop framework peptides that inhibit nicotinic acetylcholine receptors)		
GI	E C C N – P A C G R H Y S – – C*	
GIA	E C C N – P A C G R H Y S – – C G K*	
GII	E C C H – P A C G K H F S – – C*	
MI	G R C C H – P A C G K N Y S – – C*	
SI	I C C N – P A C G P K Y S – – C*	
SIA	Y C C H – P A C G K N F D – – C*	C C . . C . . . C
SII	G C C C N O A C G P B Y G – – C G T S C S	
PnIA	G C C S L P P C A A N N P D Y C*	
PnIB	G C C S L P P C A L S N P D Y C*	
Iml	G C C S D P R C A W R – – – – C*	
EI	R D O C C Y H P T C N M S N P Q I C*	
MII	G C C S N P V C H L E H S N L C*	
EpI	G C C S D P R C N M N N P D Y (SO_4)C*	
AuIB	G C C S Y P P C F A T N P D – C	
μ-conopeptides (3 loop framework that block sodium channels)		
GIIIA	R D C C T O O K K C K D R Q C K O Q R C C A*	C C . C . . . C . C C
GIIIB	R D C C T O O R K C K D R R C K O M K C C A*	
GIIIC	R D C C T O O K K C K D R R C K O L K C C A*	
PIIIA	R L C C G F O K S C R S R Q C K O H R C C*	
ω-conopeptides (4 loop framework peptides that block calcuim channels)		
GVIA	C K S O G S S C S O T S Y N C C – R S C N O Y T K R C Y	
GVIB	C K S O G S S C S O T S Y N C C – R S C N O Y T K R C Y G*	
GVIC	C K S O G S S C S O T S Y N C C – R S C N O Y T K R C*	
SVIA	C R S S G S O C G V T S I – C C G R – C – – Y R G K C T*	
SVIB	C K L K G Q S C R K T S Y D C C S G S C G R S – G K C*	C . . . C . . C C . C . . C
GVIIA	C K S O G T O C S R G M R D C C – – S C L L Y S N K C R R Y*	
GVIIB	C K S O G T O C S R G M R D C C T – S C L S Y S N K C R R Y*	
MVIIA	C K G K G A K C S R L M Y D C C T G S C R S – – G K C*	
MVIIB	C K G K G A S C H R T S Y D C C T G S C N R – – G K C*	
MVIIC	C K G K G A P C R K T M Y D C C S G S C G R R – G K C*	
MVIID	C Q G R G A S C R K T M Y N C C S G S C N R – – G R C*	
TVIA	C L S O G S S C S O T S Y N C C – R S C N O Y S R K C Y*	
δ-conopeptides (4 loop framework peptides that delay inactivation of sodium channels)		
TxVIA	W C K Q S G E M C N L L D Q N C C D G Y – C I V L V C T	
TxVIB	W C K Q S G E M C N L L D Q N C C D G Y – C I V F V C T	C . . . C . . C C . C . . C
GmVIA	V K P C R K E G Q L C D P I F Q N C C R G W N C – V L F C V	
NgVIA	S K C F S O G T F C G I K O G L C C S V R – C F S L F C I S F E	
PVIA	E A C Y A P G T F C G I K O G L C C S E F – C L P G V C F G*	
κ-conopeptides (4 loop framework pepetide that blocks Shaker potassium channels)		
PVIIA	C R I O N Q K C F Q H L D D C C S R K C N R F N K C V	C . . . C . . C C . C . . C
conantokins (helical peptides that inhibit the NMDA-glutamate receptor)		
Con-G	G E Z Z L Q Z N Q Z L I R Z K S N	
Con-T	G E Z Z Y Q K M L Z N L R Z A E V K K N A	

Amino acid sequences shown with cysteines (bold) aligned within each structural framework.
*Processed carboxyl terminal; O = hydroxyproline residue, Z = γ-carboxyglutamic acid residue. Letter prefixes indicate conopeptides from the fish hunters *C. magus* (M), *C. geographus* (G), *C. tulipa* (T), *C. striatus* (S), *C. purpurascens* (P), and *C. ermineus* (E); the mollusc hunters *C. textile* (Tx), *C. episcopatus* (Ep), *C. gloriamaris* (Gm), *C. nigropunctatus* (Ng), and *C. aulicus* (Au); and the worm hunter *C. imperialis* (Im).

The realisation that most if not all conotoxins were biologically active led us to establish chemical approaches to rapidly identify new conotoxins and confirm the presence of known conotoxins. The starting source of venom was either from the dissected venom ducts of Conidae or from the milked venom of captive species. The venom paste was then extracted with varying amounts of acetonitrile acidified with 0.1% trifluoroacetic acid. This procedure efficiently extracts most of the conotoxins present. Early research findings at the 3D Centre revealed considerable inter- and intraspecies variability in the components in cone shell venoms and also that most species contained in excess of 100 different peptides [Bingham et al., 1996]. This analysis was facilitated by the application of Ionspray mass spectrometry, which dramatically reduced the time and quantity of venom required to characterise the components of these complex mixtures [Lewis et al., 1994; Bingham et al., 1996; Jones et al., 1996]. An example of an LC/MS analysis of the peptides present in the crude venom from *Conus geographus* is given in Figure 2. From analyses of more than 30 species, it is evident that the 60+ conotoxins reported to date represent less than 0.1% of the peptides present in the venoms of Conidae.

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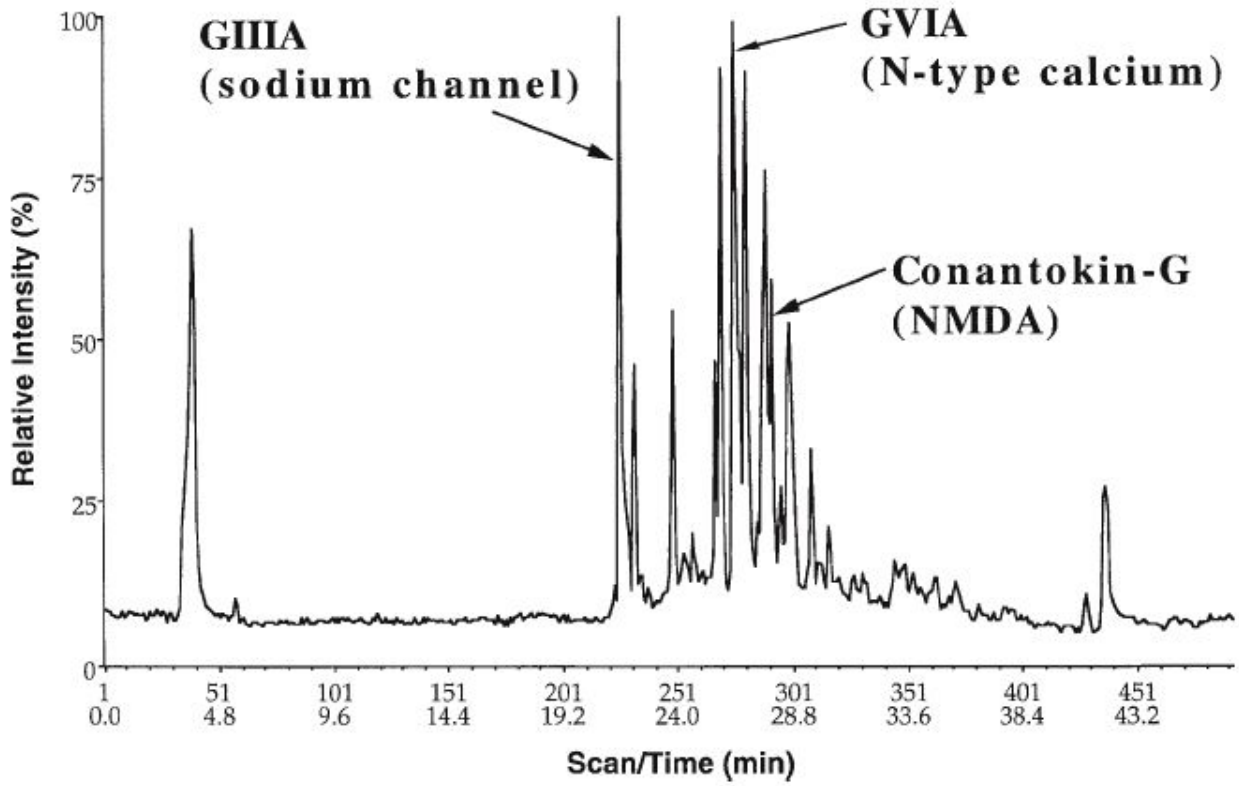

Fig. 2. LC-MS chromatogram of *Conus geographus* venom. Reverse-phase HPLC/mass spectrometry profile of the crude venom from *Conus geographus* collected on the Great Barrier Reef, Australia. Conotoxins which target sodium channels (GIIIA), N-type calcium channels (GVIA), and the NMDA receptor (conantokin G) are indicated.

HPLC/electrospray mass spectrometry analysis is generally complemented with a suite of chemical techniques to rapidly "mass profile" each crude venom. The tagging of each molecular component has facilitated the subsequent isolation and characterisation of novel peptides. Fractionation of the venom is often directed by the mass and number of disulfide bonds present in the peptide. Posttranslational modifications, which are common in cone shell venoms, are usually identified by MS/MS, enzymatic degradation/MS studies, amino acid analysis, and Edman chemistry [Loughnan et al., 1998]. Fortunately, most conopeptides are not N-terminally blocked.

The determination of disulfide bond connectivity for many conotoxins remains challenging. Classical approaches using enzymic degradation often fail, as most conotoxins are resistant to proteolysis, even with high levels of enzyme present. Success has been achieved using a reductive alkylation/Edman sequence strategy [Gray, 1993]. However, this approach occasionally fails, as the alkylation step is performed under basic conditions where scrambling may occur. Recently, we developed a more general approach that employs both mass spectrometry and Edman chemistry [Jones et al., 1996]. Briefly, the conotoxin is sequentially reduced and alkylated under acidic conditions with mass spectrometric/ HPLC analysis and Edman sequencing. For smaller peptides (e.g., the a-conotoxins), the differentially alkylated products need only be subjected to collision-induced dis sociation to locate the labeled cysteine residues and hence deduce the disulfide bond connectivity pattern.

Conotoxins are synthesised by cone shells from mRNA templates derived from toxin genes, and expressed in the venom ducts as precursor peptides. There are now numerous gene cloning techniques that can be used to isolate and characterise the precursor molecules, as a prelude to predicting the composition of the mature peptide. The mRNA can be isolated and converted to either single-stranded (ss) or double-stranded (ds) complementary DNA (cDNA). Cloning of the ds-cDNA produces a venom duct library, which can be screened with DNA probes from known toxin mature peptide sequence or precursor peptide sequence to find closely related clones. This strategy was successfully used to isolate and define the precursor structure of the w-conotoxin GVIA from a *C. geographus* library [Colledge et al., 1992]. An alternative approach is to make use of polymerase chain reaction (PCR) technologies. In this method, oligonucleotide primers homologous to known mature peptide sequence can be used to derive 5¢ leader propeptide and untranslated sequence using adaptor ligated ds-cDNA (5¢RACE). This sequence can then be used to identify conserved regions in the precursor leader sequences in which to position

oligonucleotide primers that are specific to conopeptide families. PCR using these specific primers in conjunction with a 3¢ anchor primer on venom duct ss-cDNA will produce amplified copies of the expressed peptides in that particular family (3¢ RACE). Cloning and sequencing will produce the full peptide sequence, from which the mature peptide region can be predicted. Apart from the targeted approaches of the library and PCR strategies, the complete screening of venom duct cDNA libraries in a manner similar to an EST (expressed sequence tag) strategy is quite feasible and very productive. Most conopeptide sequences are less than 1,000 nucleotides, allowing complete sequencing of each peptide gene simply by using primer sites based on the vector sequence of the clones. While the molecular cloning methods of conotoxin isolation does have a number of distinct benefits in comparison to assay directed fractionation of whole venom, they have the disadvantage of not being able to predict posttranslational modifications of the mature peptides. Conopeptides can be highly and unusually modified, such as the alpha peptide EpI, which has a sulphated tyrosine [Loughnan et al., 1998]. These modifications provide chemical alterations that may well be important in the activity of the conopeptide at the receptor target. At present, the gene structures that combine to produce a toxin peptide precursor mRNA transcript are not known. The identification of these genes and the mRNA splicing pathways that ultimately produce the highly variable toxin peptides will provide a much better understanding of toxin peptide evolution in the *Conus* species, and will undoubtedly lead to more effective strategies for library-based and PCR-based toxin peptide isolation.

Conotoxin Synthesis, folding, and purification

All conotoxins described to date, with the exception of the conantokins, contain multiple disulfide bonds. Unlike studies on other animal toxins (e.g., snakes, scorpions), both the complexity of the venom and the small quantities available (usually micrograms) preclude indepth studies on the native material. Solid phase peptide synthesis has been the most successful approach in providing significant quantities of these peptides for biological and structural studies. Most often this has been achieved through synthesis of the fully reduced polypeptide before “folding” under oxidative conditions. Although this approach yields the desired peptide, in many instances it is present in a mixture of other “wrongly” or partially folded isomers. The nonnative isomers differ solely in the connectivity of their disulfide bridges and can be difficult to separate from the native material, leading to reduced yields of pure conopeptide.

Directed Folding

Conotoxin GI is part of the a-conotoxin family and contains 13 residues with two intramolecular disulfide bridges. Various oxidative techniques on fully reduced a-conotoxin GI yield mixtures of all three potential isomers with the native isomer a-CTX GI(2-7;3-13) generally predominating. We recently described an on-resin “directed-disulfide” strategy to gain access to each isomer [Alewood, 1998]. This is illustrated in the directed synthesis of the native isomer (Fig. 3). The orthogonal protecting groups acetomidomethyl (Acm) and fluorenylmethyl (Fm) were chosen to allow stepwise regiospecific disulfide formation on the resin. Chain assembly was performed using standard Boc chemistry [Schnölzer et al., 1992] on *p*-methylbenzhydrylamine resin. The Fm group was removed and oxidised with piperidine-DMF. Deprotection and oxidation of the Acm group by iodine in DMF led to the formation of the second disulfide bond. Final HF cleavage led to deprotected “crude” conotoxin containing minor amounts of polymer. Reversed-phase HPLC analysis confirmed that only the native isomer was formed. The two nonnative isomers of a-conotoxin GI were made employing a similar strategy.

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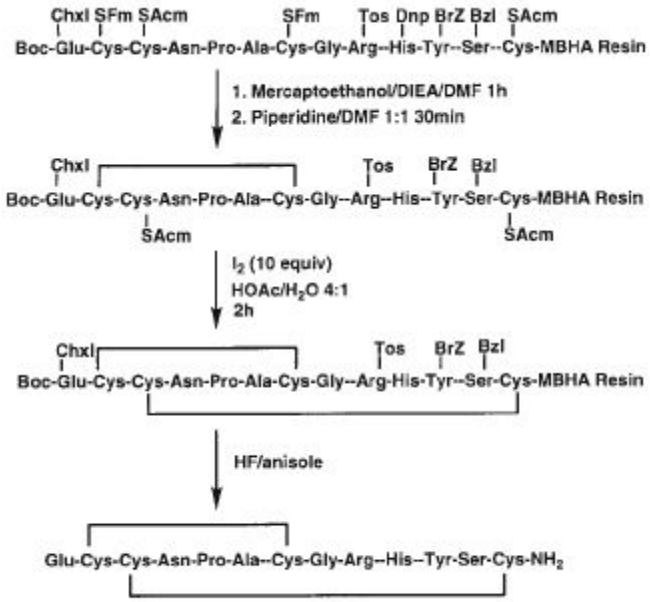

Fig. 3. Directed folding of α-conotoxin GI.

Rapid Solid Phase Peptide Synthesis (SPPS)

A bottleneck in structure–function studies of the conotoxins has been the availability of the desired mutants within a reasonable time frame. The small number of such studies reflects, in part, the difficulties in the synthesis and folding of these cysteine-rich frameworks. As such there is a pressing need to develop faster, more efficient chemistry.

In recent years, there have been efforts [Schnölzer et al., 1992; Alewood et al., 1997] by several groups to improve the speed and efficiency of SPPS. The introduction of HBTU/in situ neutralisation chemistry has allowed routine synthesis where three residues per hour are incorporated in the growing peptide chain. The further development of improved acylating agents such as HATU has opened up the possibility of more rapid synthetic procedures using HATU/Boc in situ neutralization [Alewood et al., 1997]. This is illustrated by the rapid chain assembly of the A10L mutant of PnIA conotoxin from *Conus pennaceus*, which blocks the nicotinic acetylcholine receptor. The conotoxin was assembled in a little over 1 h, worked up, and oxidised to give fully folded homogeneous material within a day.

Conotoxin Folding

Most reduced forms of native conotoxins are capable of folding efficiently. The folding/oxidation thus remains a matter of probing sufficient “folding” space so that the desired conotoxin forms uniquely or as the predominant product. Whereas many laboratories have the capacity to isolate quantities of the reduced purified precursors, their efforts at the “folding” stage have often been inadequate. This may be a direct result of not having access to native material for comparison. This is particularly important in cases where the disulfide bond connectivity of the conotoxin has not been unambiguously determined.

More specifically, the folding of w-conotoxins has caused difficulties in several laboratories where nonnative isomers have formed a significant proportion of the oxidised products. This is readily illustrated in the folding of the N-type neuronal calcium channel blocker, GVIA (Fig. 4), where the selection of inappropriate though commonly used folding conditions (trace E) led exclusively to nonnative products. Moreover, the selection of “appropriate” folding conditions (trace A) yielded almost exclusively the correctly folded native conotoxin.

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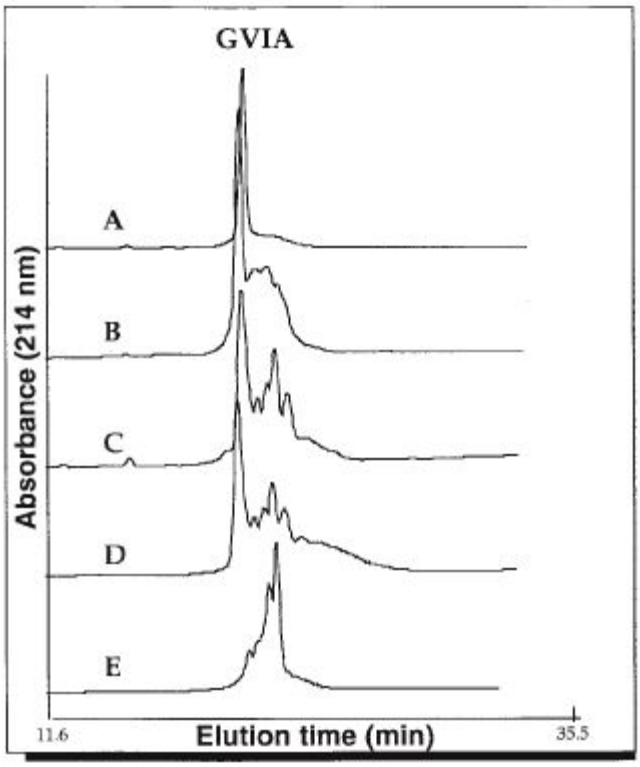

Fig. 4. Folding of ω-conotoxins GVIA under different oxidation conditions.

	Buffer	Peptide Conc (mM)	GSH/GSSG	pH
A	NH_4Oac/GnHCl (0.33M/0.5M)	0.05	100:10	7.8
B	NH_4Oac/GnHCl (0.33M/0.5M)	0.05	–	7.8
C	NH_4Oac/GnHCl (0.33M/0.5M)	0.19	100:10	7.8
D	NH_4Oac/GnHCl (0.33M/0.5M)	0.19	–	7.8
E	NaOAc (50mM)	0.05	–	7.5

*Note that the buffer used in A gave the best results and buffer E was poorest.

Structure determination of conotoxins by NMR

NMR spectroscopy is now a well-established method for structure determination of peptides and pro teins. The method relies on the measurement of a large number of distance restraints between pairs of protons. These restraints are used in a simulated annealing protocol to calculate a family of structures consistent with both the input restraints and with a force-field defining covalent geometry of atoms. Distance restraints are often supplemented with restraints on peptide backbone and sidechain dihedral angles. The distance restraints are derived from NOESY spectra and the dihedral restraints from a combination of coupling constant and NOE data. Depending on the size of the protein being studied and the complexity of the spectra, 2D, 3D, or 4D NMR methods may be required. The higher dimensional spectra (i.e., 3D or 4D) generally require uniform labeling of the protein with 15N and/or 13C isotopes so that spectral overlap may be resolved using the additional frequency dimensions associated with these NMR active nuclei, as well as the usual proton chemical shift axis.

An assumption inherent in the NMR structure determination method is that the peptide or protein adopts predominantly a single conformation in solution. For linear peptides comprising fewer than approximately 30 amino acid residues this is often not the case, with such small peptides being extremely flexible and adopting a myriad of conformations in solution. Thus, for these peptides only qualitative conclusions can be drawn about solution conformations. However, peptides which are cross-linked by disulfide bonds are more restrained in their

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conformations and are very suitable for quantitative structure determination by NMR. As indicated above, the conotoxins are rich in disulfide bonds and are hence particularly amenable to conformational analysis by NMR.

An additional advantage of conotoxins is that their small size (generally less than 30 residues) means that spectral overlap is generally not a problem, and 2D rather than 3D or 4D NMR methods are sufficient for spectral assignment and structure determination. Because isotopic labeling is not required for such studies, it is in principle possible to determine structure from native peptides extracted from venom ducts. However, in practice the amounts of material required (~1 mg) generally means that it is more convenient to synthesise the conotoxins using the methods described above.

Over the last few years we have determined the structures of more than 30 conotoxins (from all the known classes and from novel ones as yet unreported) and are using these structures in several drug design programs. From these studies, and from studies by colleagues in the literature, it has become clear that conotoxin structures fall into a limited number of families. Representatives of these structural families are summarised in Figure 5.

From these structures it can be seen that the a- conotoxins adopt a fold such that the N- and C-termini are brought into close proximity by the internal disulfide bonds, and that a short helical segment is present. By contrast, the structures of the m-, k-, and w-conotoxins are dominated by a series of loops which are superimposed on a core comprising well-defined elements of secondary structure. For the m-conotoxins these secondary structure elements include a helical region and a b-hairpin, while the k- and w-conotoxins contain a triplestranded b-sheet. The conantokins have no disulfide bonds, but adopt helical structures [Skjaerbaek et al., 1997]. We return later to a more extensive discussion of specific details of some of these structures, but emphasise here that the conotoxins clearly may be regarded as “mini-proteins” and adopt well-defined solution structures with all of the features of larger proteins. The defined presentation of amino acids on the surface of the frameworks in Figure 5 accounts for the specificity of their binding interactions and the small size of the molecules makes them valuable lead compounds in drug design applications.

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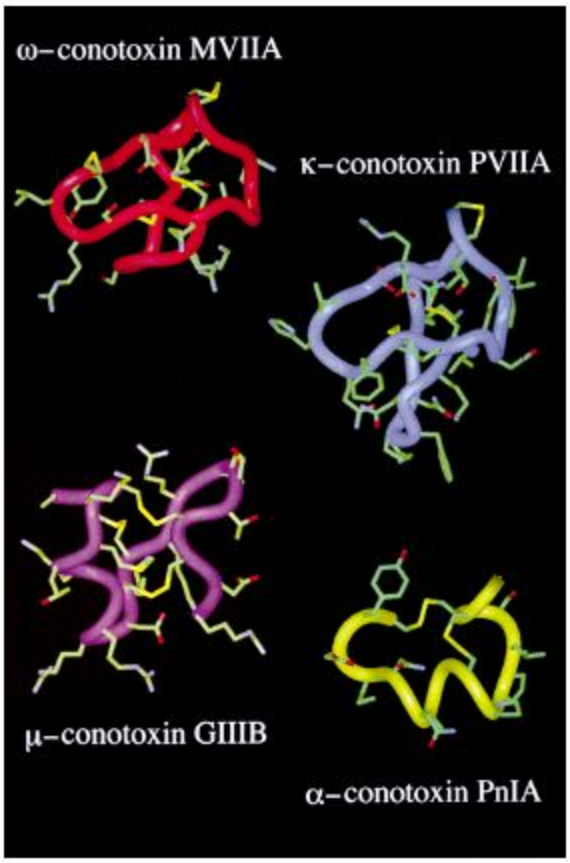

Fig. 5. Three-dimensional structures of several classes of conotoxins recently determined in our laboratory. The backbone folds are represented by tubular ribbons, with sidechains shown in stick form. Nitrogen sidechain atoms are shown in blue, oxygen atoms are in red, and the sulfur atoms of cysteine residues are in yellow.

Conotoxins Which Block the Nicotinic ACh Receptor

There are several classes of ligands that bind to the nAChR. These comprise small molecules, such as the endogenous ligand agonist acetylcholine, small peptides, including the lophotoxins and a-conotoxins, and large peptide toxins isolated from snake venoms (e.g., a- bungarotoxin). The a-conotoxins are widespread in the venoms of cone snails and have been isolated from piscivorous, molluscivorous, and vermivorous species [Gray et al., 1981; McIntosh et al., 1982, 1994; Zafaralla et al., 1988; Myers et al., 1991; Ramilo et al., 1992; Fainzilber et al., 1994; Martinez et al., 1995; Loughnan et al., 1998]. These toxins are valuable ligands for probing structure– function relationships of various nAChR subtypes as they are potent antagonists and exhibit marked selectivity between the peripheral and neuronal forms of the receptor. Typically, the a-conotoxins are 12–18 residues in length and are characterised by the presence of two conserved disulfide bonds and two loops in the peptide backbone between the cysteines. The number of amino acids in these two intra-cysteine loops varies, giving rise to the a3/5, a4/7, and a4/3 subclasses of a-conotoxins.

The affinity of a given a-conotoxin depends both on the species and the subtype of nAChR present. The first a-conotoxins discovered were found to bind to the muscle-type nAChR (e.g., a-conotoxin GI) through a highly selective interaction at the a–d over the a–g subunit interface [Hann et al., 1994; Groebe et al., 1995]. Recent studies have described the isolation and characterisation of a-conotoxins selective for *neuronal* nAChRs and the molecular basis of the interaction of these a-conotoxins with the nAChR is beginning to be revealed. For example, a-conotoxin ImI selectively targets the homomeric a7 and a9 subtypes of neuronal nAChR [Johnson et al., 1995], whereas a-conotoxin MII was found to potently block a3b2 nAChRs expressed in *Xenopus* oocytes with an IC50 of 0.5 nM [Cartier et al., 1996; Harvey et al., 1997]. The structure of MII differs significantly from the other a-conotoxins;

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however, the disulfide bonding is conserved and it has the a 4/7 spacing like a-conotoxins PnIA, PnIB, and EpI. a-Conotoxins

PnIA and PnIB from *C. pennaceus* have been reported to have a different phylogenic specificity compared to the other a-conotoxins, in that they block neuronal nAChRs in molluscs [Fainzilber et al., 1994]. However, in preliminary experiments on dissociated rat parasympathetic neurones, we found that a-conotoxins PnIA[A10L] and PnIB (0.1–1 mM) inhibit the a-bungarotoxin- sensitive component of the ACh-evoked current [Hogg et al., 1999], and identified EpI as a new selective neuronal nAChR antagonist [Loughnan et al., 1998]. We believe that the growing diversity of a- conotoxins in terms of their selectivity, not only between muscle and neuronal nAChR subtypes but between neuronal a subunits, will provide the molecular tools needed to probe and distinguish between neuronal nAChR subtypes so that their distinct function(s) can be understood.

As a result of recent studies in our laboratories, Xray crystal structures have been determined for GI [Guddat et al., 1996], PnIA [Hu et al., 1996], and PnIB [Hu et al., 1997], EpI [Hu et al., 1998], ImI and SII (unpublished structures). Combined with NMR solution structures of a similar range of a-conotoxins, they provide initial insights into the putative binding surface of these peptides. Comparison of the published structures of the a-conotoxins indicates that their backbones are superimposable. This structural consensus allows us to model differences in specificity and potency for AChRs with differences in the position of exposed sidechains in the a-conotoxins. From this analysis we will identify residues important for selectivity, allowing us to design new selective a-conotoxins. To illustrate recent work from our laboratory in this class of conotoxins, we describe studies on conotoxin GI, the first and one of the smallest a- conotoxins to be discovered, and on MII, a recently discovered member of the family with selectivity of neuronal nAChRs. The sequence and characteristics of these peptides are given in Table 2.

TABLE 2. α-Conotoxin Sequences and Specificities

Name	Sequence	Loop sizes	Species	Prey	nAChR target	Reference
GI	ECCNPACGRHYSC-NH_2	3:5	*C. geographus*	fish	α/δ	Gray et al., 1981; Groebe et al., 1997
GIA	ECCNPACGRHYSCGK-NH_2	3:5	*C. geographus*	fish		Gray et al., 1981
GII	ECCHPACGKHFSC-NH_2	3:5	*C. geographus*	fish		Gray et al., 1981
MI	GRCCHPACGKNYSC-NH_2	3:5	*C. magus*	fish	α/δ	McIntosh et al., 1982; Groebe et al., 1995
SIA	YCCHPACGKNFDC-NH_2	3:5	*C. striatus*	fish		Ramilo et al., 1992
SI	ICCNPACGPKYSC-NH_2	3:5	*C. striatus*	fish	α/δ	Zafaralla et al., 1988; Groebe et al., 1995
SII	GCCCNPACGPNYGCGTSCS	3:5:3	*C. striatus*	fish		Ramilo et al., 1992
ImI	GCCSDPRCAWRC-NH_2	4:3	*C. imperialis*	worm	α7	McIntosh et al., 1994
MII	GCCSNPVCHLEHSNLC-NH_2	4:7	*C. magus*	fish	α3β2	Cartier et al., 1997; Harvey et al., 1997
EI	RD OCCYHPTCNMSNPQIC-NH_2	4:7	*C. ermineus*	fish		Martinez et al., 1995
AuIA	GCCSYPPCFATNSDYC-NH_2	4:7	*C. aulicus*	mollusc	α3β4	Luo et al., 1998
AuIB	GCCSYPPCFATNPDC-NH_2	4:6	*C. aulicus*	mollusc	α3β4	Luo et al., 1998
AuIC	GCCSYPPCFATNSGYC-NH_2	4:7	*C. aulicus*	mollusc	α3β4	Luo et al., 1998
EpI	GCCSDPRCNMNNPDY(SO$_4$)C-NH_2	4:7	*C. episcopatus*	mollusc	α3β; α3β4	Loughnan et al., 1998
PnIA*	GCCSLPPCAANNPDYC-NH_2	4:7	*C. pennaceus*	mollusc		Fainzilber et al., 1994
PnIB*	GCCSLPPCALSNPDYC-NH_2	4:7	*C. pennaceus*	mollusc	α7	Fainzilber et al., 1994
αA-PIVA	GCCCSYONAACHOCSCKDRGSYCGQ-NH_2	7:2:1:6	*C. purpurascens*	fish	α/δ; α/γ	Hopkins et al., 1995
αA-EIVA	GCCGPYONAACHOCGCKVGROOYCDROSGG-NH_2	7:2:1:7	*C. ermineus*	fish	α/δ; α/γ	Jacobsen et al., 1997
αA-EIVB	GCCGKYONAACHOCGCTVGROOYCDROSGG-NH_2	7:2:1:7	*C. ermineus*	fish	α/δ; α/γ	Jacobsen et al., 1997

*PnIA and PnIB have been shown to target neuronal nAChRs of molluscs and, more recently, PnIA[A10L] and PnIB have been reported to block the mammalian α7 nAChR subunit [Hogg et al., 1999].

Conotoxin GI

Our interest in GI has focused on its use as a model to explore conformational diversity resulting from disulfide bond engineering. As already noted, conotoxins are characterised by their particularly high content of cysteine, with the cysteine residues almost invariably connected in pairs to form disulfide bonds. In peptide toxins, even more so than in larger proteins, these disulfide bonds have a crucial bearing on three-dimensional structure and function. As the number of cysteine residues in a peptide increases, the number of ways of connecting the cysteines in disulfide bonds increases dramatically, leading to a large number of potential isomers. It is interesting and highly significant that invariably only one of the possible isomers occurs naturally, i.e., venoms do not nor mally contain different isomers of the same conotoxins with different connections of the disulfide bonds. However, using solid phase chemical methods it is possible to selectively produce each of the individual disulfide bond isomers. As noted in a section above, we used this approach to synthesise all three possible disulfide bond isomers of the a-conotoxin GI and have determined their structures [Gehrmann et al., 1998]. We refer to the three isomers as GI(2-7;3-13), GI(2-13;3-7), and GI(2-3;7-13).

The structural findings may be summarised by noting that the native connectivity of the four constituent cysteine residues produces a significantly more stable and well defined structure than either of the two alternative

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arrangements of the disulfide bonds [Gehrmann et al., 1998]. A single solution conformation was detected for the native isomer, GI(2-7;3-13), which consists primarily of a distorted 310 helix from residues 5 to 11. The two nonnative forms exhibit multiple conformations in solution, with the major populated forms being different in structure both from each other and with the native form. We concluded that the disulfide bonds in GI play a major role in determining both the structure and stability of the peptide. A trend for increased conformational flexibility was observed in the order GI(2-7;3-13) < GI(2- 13;3-7) < GI(2-3;7-13).

Interest in making nonnative isomers arises because peptide analogues are widely regarded as valuable drug leads, and in recent years there has been much effort directed towards the development of peptide libraries. It has been of particular interest to develop methods to increase the surface variability of peptides because the diversity of peptide libraries are, to some extent, limited by the use of the 20 natural amino acids. The study described above shows that the use of alternative disulfide bond connectivities provides another way of altering molecular conformations without modifying the sequence.

Conotoxin MII

The recently identified a-conotoxin MII from *C. magus* belongs to the a4/7 subclass and is a potent and highly specific blocker of mammalian neuronal nAChRs composed of a3b2 subunits. MII was first reported by Cartier et al. [1996] following the electrophysiological screening of RP-HPLC fractions of duct venom against cloned nAChRs expressed in *Xenopus* oocytes. We independently isolated and characterised MII as part of a comprehensive study of the milked venom of *C. magus* and recently reported its three-dimensional structure [Hill et al., 1998].

The molecule folds into a highly compact globular structure consisting of a central region of a-helix and a series of overlapping b-turns at the N- and Ctermini. The a-helix comprising residues 6–12 exhibits two turns and is amphipathic, with Cys8, His9, Glu11, and His12 on one side and Pro6, Val7, and Leu10 on the other. Remarkably, the hydrophobic residues of the a-helix are more exposed to the solvent than the charged/hydrophilic residues. However, this is consistent with the fact that MII is more hydrophobic when oxidised than in the reduced form. Hydrophilic residues on the surface include Ser4, Asn5, and residues Glu11, His12, Ser13, and Asn14. The latter patch, comprising residues with both polar and charged groups (Glu11-Asn14), may be responsible for initial recognition by the nAChR, with further stabilisation of binding provided by the proximal hydrophobic residues. Analysis of the solvent accessibility of individual residues provides support for Pro6, Val7, Leu10, Glu11, and Asn 14 as potential residues for interaction with the nAChR as they are highly solvent exposed [Hill et al., 1998].

Sodium Channel Binding Conotoxins

The piscivorous cone snail, *C. geographus*, produces polypeptide neurotoxins that specifically inhibit skeletal muscle and eel electroplax sodium channels [Sato et al., 1983; Cruz et al., 1985; Yanagawa et al., 1988; Moczydlowski et al., 1986]. These toxins, the m-conotoxins and conotoxin GS, are attractive probes of sodium channel structure because of their high binding affinity and ability to discriminate between the skeletal muscle and neuronal and cardiac channel isoforms [Yanagawa et al., 1988; Moczydlowski et al., 1986; Ohizumi et al., 1986; Chen et al., 1992]. It is remarkable that while these peptides belong to the same pharmacological class they have different structural frameworks, as illustrated in Table 1.

The m-conotoxins, a family of highly basic 22-residue polypeptides (GIIIA, GIIIB, and GIIIC), contain six cysteine residues which are paired in a 1–4, 2–5, 3–6 pattern to form three intramolecular disulfide bonds and a three-loop framework. Conotoxin GS has a strikingly different sequence and is 50% larger than the m- conotoxins. This polypeptide contains six cysteine residues arranged in a similar 1–4, 2–5, 3–6 pattern [Nakao et al., 1995]; however, differences in the spacings between cysteine residues results in a four-loop framework rather than a three-loop framework. Despite the low sequence identity, conotoxin GS binds competitively with m- conotoxin GIIIA, suggesting overlapping binding sites on the extracellular surface of skeletal muscle and eel electroplax sodium channels [Yanagawa et al., 1988].

Conotoxin GIIIB

GIIIB adopts a compact structure [Hill et al., 1996] consisting of a distorted 310-helix, a small b-hairpin, a cishydroxyproline, and several turns. The molecule is stabilised by three disulfide bonds, two of which connect the helix and the b-hairpin, forming a structural core with similarities to the CSab motif [Cornet et al., 1995]. This motif is common to several families of small proteins, including scorpion toxins and insect defensins. Other structural features of GIIIB include the presence of eight arginine and lysine sidechains that project into the solvent in a radial

orientation relative to the core of the molecule. These cationic sidechains form potential sites of interaction with anionic sites on sodium channels. The global fold is similar to that reported for m- conotoxin GIIIA, and together the structures provide a basis for further understanding of the structure–activity relationships of the m-conotoxins and for their binding to skeletal muscle sodium channels.

Conotoxin GS

The three-dimensional structure of conotoxin GS [Hill et al., 1997] consists of a compact, disulfide-bonded core from which several loops and the C-terminus project. The main element of secondary structure is a doublestranded antiparallel b-sheet comprising residues 17–20 and 26–29 connected by a turn involving residues 21–25 to give a b-hairpin structure. A further peripheral b-strand involving residues 7–9 is almost perpendicular to the b- hairpin, with only Ser7 hydrogen-bonded to the central b-strand forming an isolated b-bridge.

GS is unusual in that it contains the posttranslationally modified residue g–carboxy glutamic acid. To investigate the role of Gla32 in this polypeptide, an analog [Glu32]conotoxin GS was synthesised and the NMR spectra compared with those of conotoxin GS. The chemical shift differences for the backbone Ha and NH protons of conotoxin GS and [Glu32]conotoxin GS were small (£0.05 ppm), suggesting that the backbone conformation of the two peptides is essentially identical. Several other parameters, including the observed NOEs, 3JNH-Ha coupling constants and amide exchange rates are similar, providing further evidence of conserved structure in these peptides. This suggests that the Gla residue does not play a role in modulating the three-dimensional structure of conotoxin GS.

As the sequence and structure of conotoxin GS is quite different from the m-conotoxins, it provides a valuable new probe for further characterisation of sodium channel geometry. The structure of conotoxin GS will facilitate the design of analogues to define the binding surface and to undertake complementary mutagenesis on the sodium channel to identify the interacting residues. These experiments with conotoxins may prove as useful in modeling the outer vestibule of sodium channels as the peptide toxins from scorpions have been for potassium channels.

Calcium Channel Blocking Conotoxins

The w-conotoxins are a set of structurally related peptides that have a wide range of specificities for different subtypes of the voltage-sensitive calcium channel (VSCC). To understand their VSCC subtype differentiation, we studied the structure of two naturally occurring w-conotoxins, MVIIA (specific to N-type VSCCs) and SVIB (specific to P/Q-type) and a synthetic hybrid, SNX- 202, which has altered specificities to both VSCC subtypes [Nielsen et al., 1996]. The secondary structures of the three peptides are almost identical, consisting of a triple-stranded b-sheet and several turns. The three-dimensional structures of SVIB and MVIIA are likewise quite similar, but some subtle differences are manifested as orientational differences between two key loops.

A remarkable feature of the six cysteine / four-loop framework exemplified by the w-conotoxins is the presence of a cystine knot within the structures. This motif consists of an embedded loop in the structure formed by two of the disulfide bonds and their connecting backbone segments. This loop is penetrated by the third disulfide bond in a remarkable example of Nature’s engineering designs.

Although the structural rigidity of the core of MVIIA is apparently assured by the knotted disulfide structure, we used NMR to probe for possible conformational flexibility in the exposed loops. As indicated above, it is important be aware of potential conformational changes that might affect receptor binding. In the case of MVIIA, the Ha shifts were found to be similar in a range of solvents, indicating that there are no solventinduced changes in structure.

From the above structural studies and a large number of other studies of molecules within this family it is apparent that the w-conotoxins form a consensus structure despite differences in sequence and VSCC subtype specificity. This indicates that the w-conotoxin macrosites for the N/P/Q-subfamily of VSCCs are related, with specificity for receptor targets being conferred by the positions of functional sidechains on the surface of the peptides.

As mentioned earlier, the w-conotoxins have attracted the most interest for potential pharmaceutical applications. Indeed, conotoxin MVIIA is currently in clinical trial for the treatment of chronic pain. Structural studies of the type described above are likely to lead to the development of second-generation analogues which may overcome some of the side effects of MVIIA itself.

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Potassium Blocking Conotoxins

k-PVIIA is a 27-residue polypeptide isolated from the venom of *C. purpurascens* and is the first member of a new class of conotoxins that block potassium channels. By comparison to other ion channels of eukaryotic cell membranes, voltage-sensitive potassium channels are relatively simple and methodology has been developed for mapping their interactions with small peptide toxins. PVIIA, therefore, is a valuable new probe of potassium channel structure. In a recent study, we determined the solution structure and mode of channel binding of PVIIA [Scanlon et al., 1997] and this forms the basis for mapping the interacting residues at the conotoxin–ion channel interface.

The three-dimensional structure of PVIIA resembles the triple-stranded b-sheet / cystine knot motif formed by a number of toxic and inhibitory peptides, including the w-conotoxins and conotoxin GS, as described above. Subtle structural differences, however, predominantly in loops 2 and 4, are observed between PVIIA and other conotoxins with similar structural frameworks. Electrophysiological binding data suggest that PVIIA blocks K+ channel currents by binding in a voltage-sensitive manner to the external vestibule and occluding the pore. Comparison of the electrostatic surface of PVIIA with that of the well-characterised potassium channel blocker charybdotoxin suggested a likely binding orientation for PVIIA. Although the structure of PVIIA is considerably different from that of the aK scorpion toxins, it has a similar mechanism of channel blockade. On the basis of a comparison of the structures of PVIIA and charybdotoxin, we suggested that Lys 19 of PVIIA is the residue responsible for physically occluding the pore of the potassium channel.

Common Structural Frameworks.

From the studies described above it has become clear that conotoxins with the six cysteine / four-loop framework are the most abundant group of peptides isolated from *Conus* venoms so far. This structural class encompasses at least five known pharmacological classes: w-conotoxin calcium channel blockers, d-conotoxins which inhibit the inactivation of sodium channels, k- conotoxin PVIIA which blocks potassium channels, the sodium channel blocker conotoxin GS, and two peptides recently found in *C. marmoreus* that affect both sodium and calcium currents [Myers et al., 1993; Cruz, 1996; Terlau et al., 1996]. The solution structures of several of these classes have now been determined, including the w-conotoxins, k-conotoxin, and GS, and all contain a triple-stranded antiparallel b-sheet with +2*x*, -1 topology and cystine knot motif common to that observed in a number of toxic and inhibitory peptides [Pallaghy et al., 1994; Narasimhan et al., 1994].

Thus, there are now many examples where one structural framework is associated with different pharmacological activities. Interestingly, the converse also occurs; that is, the same pharmacological activity may be associated with completely different structural frameworks, as demonstrated, for example, with the studies described above on the m-conotoxins and conotoxin GS.

Conclusions

Conotoxins provide a vast library of peptides with unique abilities to discriminate among types and subtypes of ion channels in a manner that is unmatched by the typical small molecule drugs which dominate the pharmaceutical industry. In addition, cone venom peptides are small and inherently stable, making them ideal leads for peptide therapeutics, especially ion channel therapeutics. The high structural resolution now obtained with modern NMR spectroscopy and X-ray crystallography provides emerging opportunities to use conotoxins as templates for the design of smaller peptidomimetics that incorporate the selectivity and potency of conotoxins. Because of its selectivity and potency, w-conotoxin MVIIA (Ziconotide) is being developed as a drug for the treatment of chronic pain. Conotoxins continue to be discovered that define new pharmacological targets. With improvement in methods of delivering peptides, it is anticipated that conopeptides can be modified for effective oral delivery.

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References

Alewood PF. 1998. Conotoxins as molecular templates for drug design. In: Ramage R, Epton R, editors. Peptides. Bodwin, UK: Mayflower Scientific. p 183–186.

Alewood PF, Alewood D, Miranda L, Love S, Meutermans W, Wilson D. 1997. Rapid *in situ* neutralization protocols for Boc and Imoc solid phase chemistries. Methods Enzymol 289:14–29.

Bingham J-P, Jones A, Lewis RJ, Andrews PR, Alewood PF. 1996. *Conus* venom peptides (conopeptides): inter-species, intra-species and within individual variation revealed by ionspray mass spectrometry. In: Lazarovici P et al., editors. Biochemical aspects of marine pharmacology. Alaken Inc., Colorado. p 13–27.

Cartier GE, Yoshikami D, Gray WR, Luo S, Olivera BM, McIntosh JM. 1996. A new a-conotoxin which targets a3b2 nicotinic acetylcholine receptors. J Biol Chem 271:7522–7528.

Chen L-Q, Chahine M, Kallen RG, Barchi RL, Horn R. 1992. Chimeric study of sodium channels from rat skeletal and cardiac muscle. FEBS Lett 309:253–257.

Colledge CJ, Hunsperger JP, Imperial JS, Hillyard DR. 1992. Precursor structure of w-conotoxin GVIA from a cDNA clone. Toxicon 30:111–1116.

Colquhoun LM, Patrick JW. 1997. Pharmacology of neuronal nicotinic acetylcholine receptor subtypes. Adv Pharmacol 39:191–220.

Cornet B, Bonmatin J-M, Hetru C, Hoffman JA, Ptak M, Vovelle F. 1995. Refined three-dimensional solution structure of insect defensin A. Structure 3:435–448.

Cruz LJ. 1996. Primary structural motifs of *Conus* peptides. In: Singh BR, Tu AT, editors. Natural toxins II. New York: Plenum Press. p 155–167.

Cruz LJ, Gray WR, Olivera BM, Zeikus RD, Kerr L, Yoshikami D, Moczydlowski E. 1985. *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. J Biol Chem 260:9280–9288.

Doyle DA, Cabral JM, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT, MacKinnon R. 1998. The structure of the potassium channel: molecular basis of K+ conduction and selectivity. Science 280:69–77.

Fainzilber M, Hasson A, Oren R, Burlingame AL, Gordon D, Spira ME, Zlotkin E. 1994. New mollusc-specific alpha-conotoxins block *Aplysia* neuronal acetylcholine receptors. Biochemistry 33:9523– 9529.

Gray WR. 1993. Disulfide structures of highly bridged peptides: a new strategy for analysis. Protein Sci 2:1732–1748.

Gray WR, Luque A, Olivera BM, Barrett J, Cruz LJ. 1981. Peptide toxins from *Conus geographus* venom. J Biol Chem 256: 4734–4740.

Gehrmann J, Alewood PF, Craik DJ. 1998. Structure determination of the three disulfide bond isomers of a-conotoxin GI: a model for the role of disulfide bonds in structural stability. J Mol Biol 278:401–415.

Groebe DR, Dumm JM, Levitan ES, Abramson SN. 1995. a- Conotoxins selectively inhibit one of the two acetylcholine binding sites of nicotinic receptors. Mol Pharmacol 48:105–111.

Guddat LW, Martin JL, Shan L, Edmundson AB, Gray WR. 1996. Three-dimensional structure of the a-conotoxin GI at 1.2 Å resolution. Biochemistry 35:11329–11335.

Hann RM, Pagon OR, Eterovic VA. 1994. The a-conotoxins GI and MI distinguish between the nicotinic acetylcholine receptor agonist sites while SI does not. Biochemistry 33:14058–14063.

Harvey SC, McIntosh JM, Cartier GE, Maddox FN, Luetje CW. 1997. Determinants of specificity for a-conotoxin MII on a3b2 neuronal nicotinic receptors. Mol Pharmacol 51:336–342.

Hill JM, Alewood PF, Craik DJ. 1996. Three-dimensional solution structure of m-conotoxin GIIIB, a specific blocker of skeletal muscle sodium channels. Biochemistry 35:8824–8835.

Hill JM, Alewood PF, Craik DJ. 1997. Solution structure of the sodium channel antagonist conotoxin GS: a new molecular caliper for probing sodium channel geometry. Structure 5:571–583.

Hill JM, Oomen CJ, Miranda LP, Bingham J-P, Alewood PF, Craik DJ. 1998. Three-dimensional solution structure of a-conotoxin MII by NMR spectroscopy: effects of solution environment on helicity. Biochemistry 37:15621–15630.

Hogg RC, Lewis RJ, Adams DJ. 1999. a-Conotoxin analogue, PnIA[A10L], and a-bungarotoxin block the same component of nicotinic ACh-induced current in rat parasymapthetic neurons. Proc Aust Neurosci Soc 10:152.

Hopkins C, Grilley M, Miller C, Shon K-J, Cruz LJ, Gray WR, Dykert J, Rivier J, Yoshikami D, Olivera BM. 1995. A new family of *Conus* peptides targed to the nicotinic acetylcholine receptor. J Biol Chem 270:22361–22367.

Hu S-H, Gehrmann J, Guddat LW, Alewood PF, Craik DJ, Martin JL. 1996. The 1.1 Å crystal structure of the neuronal acetylcholine receptor antagonist, a-conotoxin PnIA from *Conus pennaceus*. Structure 4:417–423.

Hu S-H, Gehrmann J, Alewood PF, Craik DJ, Martin JL. 1997. Crystal structure at 1.1 Å resolution of a-conotoxin PnIB: comparison with a-conotoxins PnIA and GI. Biochemistry 36:11323–11330.

Drug Development Research (1999) 46 (3-4), 219-234. doi: 10.1002/(SICI)1098-2299(199903/04)46:3/4<219::AID-DDR7>3.0.CO;2-S

Hu S-H, Loughnan M, Miller R, Weeks CM, Blessing RH, Alewood PF, Lewis RJ, Martin JL. 1998. The 1.1 Å resolution crystal struc ture of [Tyr15]EpI, a novel a-conotoxin from *Conus episcopatus*, solved by direct methods. Biochemistry 37:11425–11433.

Jacobsen R, Yoshikami D, Ellison M, Martinez J, Gray WR, Cartier GE, Shon K-J, Groebe DR, Abramson SN, Olivera BM, McIntosh JM. 1997. Differential targeting of nicotinic acetylcholine receptors by novel aA-conotoxins. J Biol Chem 272:22531–22537.

Jan LY, Jan YN. 1997. Cloned potassium channels from eukaryotes and prokaryotes. Annu Rev Neurosci 20:91–123.

Johnson DS, Martinez J, Elgoyhen AB, Heinemann SF, McIntosh JM. 1995. a-Conotoxin Im1 exhibits subtype-specific nicotinic acetylcholine receptor blockade: preferential inhibition of homomeric a7 and a9 receptors. Mol Pharmacol 48:194–199.

Jones A, Bingham J-P, Gehrmann J, Bond T, Loughnan M, Atkins A, Lewis RJ, Alewood PF. 1996. Isolation and characterisation of conopeptides by high-performance liquid chromatography combined with mass spectrometry and tandem mass spectrometry. Rapid Commun Mass Spectrom 10:138–143.

Kaiser SA, Soliakov L, Harvey SC, Luetje CW, Wonnacott S. 1998. Differential inhibition by a-conotoxin-MII of the nicotinic stimulation of [3H]dopamine release from rat striatal synaptosomes and slices. J Neurochem 70:1069–1076.

Kulak JM, Nguyen TA, Olivera BM, McIntosh JM. 1997. a-Conotoxin MII blocks nicotine-stimulated dopamine release in rat striatal synaptosomes. J Neurosci 17:5263–5270.

Lewis RJ, Bingham J–P, Jones A, Alewood PF. 1994. Analysis of *Conus* venoms by ionspray mass spectrometry. Australasian Biotech 4:298–300.

Loughnan M, Bond T, Atkins A, Cuevas J, Adams DJ, Broxton NM, Livett BG, Down JG, Jones A, Alewood PF, Lewis RJ. 1998. a- Conotoxin EpI, a novel sulfated peptide from *Conus episcopatus* that selectively targets neuronal nicotinic acetylcholine receptors. J Biol Chem 273:15667–15674.

Luo S, Kulak JM, Cartier GE, Jacobsen RB, Yoshikami D, Olivera BM, McIntosh JM. 1998. a-Conotoxin AuIB selectively blocks a3b4 nicotinic acetylcholine receptors and nicotine-evoked norepinephrine release. J Neurosci 18:8571–8579.

Martinez JS, Olivera BM, Gray WR, Craig AG, Groebe DR, Abramson SN, McIntosh JM. 1995. a-Conotoxin EI, a new nicotinic acetylcholine receptor antagonist with novel selectivity. Biochemistry 34:14519–14526.

McGehee DS, Role LW. 1995. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu Rev Physiol 57:521–546.

McIntosh JM, Cruz LJ, Hunkapiller MW, Gray WR, Olivera BM. 1982. Isolation and structure of a peptide toxin from the marine snail *Conus magus*. Arch Biochem Biophys 218:329–334.

McIntosh JM, Yoshikami D, Mahe E, Nielsen DB, Rivier JE, Gray WR, Olivera, BM. 1994. A nicotinic acetylcholine receptor ligand of unique specificity, a-conotoxin ImI. J Biol Chem 269:16733– 16739.

Miller C. 1995. The charybdotoxin family of K+-channel blocking peptides. Neuron 15:5–10.

Moczydlowski E, Olivera BM, Gray WR, Strichartz GR. 1986. Discrimination of muscle and neuronal Na-channel subtypes by binding competition between [3H]saxitoxin and m-conotoxins. Proc Natl Acad Sci USA 83:5321–5325.

Myers RA, Zafaralla GC, Gray WR, Abbott J, Cruz LJ, Olivera BM. 1991. a-Conotoxins, small peptide probes of nicotinic acetylcholine receptors. Biochemistry 30:9370–9377.

Myers RA, Cruz LJ, Rivier JE, Olivera BM. 1993. *Conus* peptides as chemical probes for receptors and ion channels. Chem Rev 93:1923– 1936.

Nakao M, Nishiuchi Y, Nakata M, Watanabe TX, Kimura T, Sakakibara S. 1995. Synthesis and disulfide structure determination of conotoxin GS, a g-carboxyglutamic acid-containing neurotoxic peptide. Lett Pept Sci 2:17–26.

Narasimhan L, Singh J, Humblet C, Guruprasad K, Blundell T. 1994. Snail and spider toxins share a similar tertiary structure and 'cystine motif.' Nat Struct Biol 1:850–852.

Nielsen KJ, Thomas L, Lewis RJ, Alewood PF, Craik DJ. 1996. A consensus structure for w-conotoxins with different selectivities for voltage-sensitive calcium channel subtypes: comparison of MVIIA, SVIB and SNX-202. J Mol Biol 263:297–310.

Noda M, Shimizu S, Tanabe T, Takai T, Kayano T, Ikeda T, Takahashi H, Nakayama H, Kanaoka Y, Minamino N, Kangawa K, Matsuo H, Raftery MA, Hirose T, Inayama S, Hayashida H, Miyata T, Numa S. 1984. Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. Nature 312:121–127.

Ohizumi Y, Nakamura H, Kobayashi J, Catterall WA. 1986. Specific inhibition of [3H]saxitoxin binding to skeletal muscle sodium channels by geographutoxin II, a polypeptide channel blocker. J Biol Chem 261:6149–6152.

Olivera BM, Rivier J, Clark C, Ramilo C, Corpuz GP, Abogadie FC, Mena EE, Woodward SR, Hillyard DR, Cruz LJ. 1990. Diversity of *Conus* neuropeptides. Science 249:257–263.

Olivera BM, Miljanich G, Ramachandran J, Adams ME. 1994. Calcium channel diversity and neurotransmitter release: the w- conotoxins and w-agatoxins. Annu Rev Biochem 63:823–867.

Drug Development Research (1999) 46 (3-4), 219-234. doi: 10.1002/(SICI)1098-2299(199903/04)46:3/4<219::AID-DDR7>3.0.CO;2-S

Pallaghy PK, Nielsen KJ, Craik DJ, Norton RS. 1994. A common structural motif incorporating a cystine knot and a triple-stranded b-sheet in toxic and inhibitory polypeptides. Protein Sci 3:1833–1839.

Picciotto MR, Zoli M, Rimondini R, Léna C, Marubio LM, Pich EM, Fuxe K, Changeux J-P. 1998. Acetylcholine receptors containing the b2 subunit are involved in the reinforcing properties of nicotine. Nature 391:173–177.

Poth K, Nutter TJ, Cuevas J, Parker MJ, Adams DJ, Luetje CW. 1997. Heterogeneity of nicotinic receptor class and subunit mRNA expression among individual parasympathetic neurons from rat intracardiac ganglia. J Neurosci 17:586–596.

Ramilo CA, Zafaralla GC, Nadasdi L, Hammerland LG, Yoshikami D, Gray WR, Kristipati R, Ramachandran J, Miljanich G, Olivera BM, Cruz LJ. 1992. Novel a- and w-conotoxins from *Conus striatus* venom. Biochemistry 31:9919–9926.

Sangameswaran L, Delgado SG, Fish LM, Koch BD, Jakeman LB, Stewart GR, Sze P, Hunter JC, Eglen RM, Herman RC. 1996. Structure and function of a novel voltage-gated, tetrodotoxin-resistant sodium channel specific to sensory neurons. J Biol Chem 271:5953– 5956.

Sato S, Nakamura H, Ohizumi Y, Kobayashi J, Hirata Y. 1983. The amino acid sequences of homologous hydroxyproline-containing myotoxins from the marine snail *Conus geographus* venom. FEBS Lett 155:277–280.

Scanlon MJ, Naranjo D, Thomas L, Alewood, PF, Lewis RJ, Craik DJ. 1997. The structure of novel potassium channel toxin, k-conotoxin PVIIA, from the venom of *Conus purpurascens*: implications for the mechanism of channel block. Structure 5:1585–1597.

Schnölzer M, Alewood P, Jones A, Alewood D, Kent SBH. 1992. *In situ* neutralization in Boc-chemistry solid phase peptide synthesis. Int J Pept Protein Res 40:180–193.

Shon K-J, Olivera BM, Watkins M, Jacobsen RB, Gray WR, Floresca CZ, Cruz LJ, Hillyard DR, Brink A, Terlau H, Yoshikami D. 1998. m-Conotoxin PIIIA, a new peptide for discriminating among tetrodotoxin-sensitive Na channel subtypes. J Neurosci 18:4473–4481.

Skjaerbaek N, Nielsen KJ, Lewis RJ, Alewood P, Craik DJ. 1997. Determination of the solution structures of conantokin-G and conantokin-T by CD and NMR spectroscopy. J Biol Chem 272:2291–2299.

Terlau H, Shon K-J, Grilley M, Stocker M, Stühmer W, Olivera BM. 1996. Strategy for rapid immobilisation of prey by a fish-hunting marine snail. Nature 381:148–151.

Unwin N. 1998. The nicotinic acetylcholine receptor of the *Torpedo* electric ray. J Struct Biol 121:181–190.

Watson MJ, Lewis RJ, Adams DJ. 1998. Effects of synthetic m- conotoxins on voltage-dependent sodium currents in rat sensory neurones. Proc Aust Physiol Pharmacol Soc 29:326P.

Yanagawa Y, Abe T, Satake M, Odani S, Suzuki J, Ishikawa K. 1988. A novel sodium channel inhibitor from *Conus geographus*: purification, structure, and pharmacological properties. Biochemistry 27:6256–6262.

Zafaralla GC, Ramilo C, Gray WR, Karlstrom R, Olivera BM, Cruz LJ. 1988. Phylogenetic specificity of cholinergic ligands: a-conotoxin SI. Biochemistry 27:7102–7105.