Scientific paper

The Evolution of αD-Conopeptides Targeting Neuronal Nicotinic Acetylcholine Receptors

Dietrich Mebs,[1]* Dušan Kordiš,[2] Yvonne Kendel[1] and Silke Kauferstein[1]

[1] *Institute of Legal Medicine, University of Frankfurt, Kennedyallee 104, D-60596 Frankfurt, Germany*

[2] *Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Jamova Street 39, SI-1000 Ljubljana, Slovenia.*

** Corresponding author: E-mail: mebs@em.uni-frankfurt.de*

Received: 12-05-2011

Dedicated to the memory of Professor Franc Gubenšek

Abstract

Venoms of the marine cone snails (*Conus* spp.) consist of numerous proteins and peptides showing a wide variety of biological activities such as on ion-channels and receptors. Peptides acting on neuronal nicotinic acetylcholine receptors belong to several peptide superfamilies including the recently described αD-conopeptides which are homodimers of identical peptides with 47–49 amino acids. Among the venom glands of 27 *Conus* species analyzed by cDNA cloning, precursors of αD-conopeptides were identified in four species only: *C. betulinus, C. capitaneus, C. mustelinus,* and *C. vexillum*. Phylogenetic analysis of the relationships among the αD-conopeptides revealed that they belong to clades, which are characterized by an AVV- and EMM-motif in the signal peptide sequence.

Keywords: *Conus,* conopeptides, αD-conopeptide superfamily, neuronal nicotinic acetylcholine receptor

1. Introduction

The marine snails of the genus *Conus* (family: Conidae) use a powerful venom to subdue prey such as fish, worms or other snails, and to use it also for defense. Each venom represents a cocktail of numerous peptides acting on a variety of receptors and ion channels.[1,2] Among them, a group of peptides are targeting nicotinic acetylcholine receptors (nAChRs), which they effectively block causing almost immediate paralysis of the prey. According to their structural properties, they have been assigned to several superfamilies: A- (α- and αA-conopeptides), M-(ψ- conopeptides), S- (αS- conopeptides), C-(αC- conopeptides), L- (lt-conopeptides) and D-superfamily (αD-conopeptides).[1,3–7] Peptides of the A- and L-superfamily such as the small α-conopeptides consist of 12 to 15 amino acids in a chain cross-linked by two disulfide bridges. Depending on the number of amino acids between the third and fourth cysteine residue, they have been classified into various subtypes which exhibit different activities to nAChRs, muscle as well as neuronal subtypes.[7,8] The larger αA-conopeptides (30 amino acids and three disulfide bridges) inhibit specifically muscle nAChRs like the αC- and ψ- conopeptides which act also as non-competitive antagonists.[4,9–11] The αS- conopeptides block muscle as well as various neuronal nAChRs subtypes.[12]

The recently described αD-conopeptides selectively inhibit α7 and β2 containing neuronal nAChRs only.[5,6,13] They represent homodimers of identical peptides with 47–49 amino acid residues and show a characteristic arrangement of 10 cysteines. These novel conopeptides are the major constituents of venoms from *Conus capitaneus, C. mustelinus* and *C. vexillum*.[5,6,13] In the present paper the screening of 27 *Conus* species for the occurrence of αD-conopeptides in their venom gland using cDNA cloning methods and the evolutionary implications are described.

2. Materials and Methods

2. 1. Materials

Specimens of *Conus betulinus, C. capitaneus, C. circumcisus, C. ebraeus, C. flavidus, C. generalis, C. geographus, C. imperialis, C. litoglyphus, C. litteratus, C. marmoreus, C. miles, C. mustulinus, C. planorbis, C. striatus, C. terebra, C. tesselatus, C. textile, C. vexillum, C. virgo, C. vulpinus* were collected in the reefs of Olango Island, Cebu (Philippines), specimens of *C. catus* and *C. distans* of Takapoto Island, Polynesia, and *C. coelinae, C. eburneus, C. leopardus* around Chesterfield Island, New Caledonia. The venom ducts of one or two specimens of each species were dissected, immediately placed in RNAlater® (Sigma-Aldrich, St. Louis, MO, USA) and stored at –20 °C.

2. 2. Preparation of Total RNA, cDNA Cloning and Sequencing

Venom duct total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was prepared by reverse transcription of the RNA using the Omniscript RT Kit (QIAgen, Chatsworth, CA, USA). To determine the cDNA sequence of the peptides the forward degenerated primer (5'-ACN CCN GGN TCN AAR TGG GGN-3') corresponding to the amino acid residues TPGSKWG, which was shown to be a part of the peptide sequence of VxXIIA by Loughnan et al.[12], paired with an abridged universal amplification primer (Qa) devoid of the poly dT tail (5'-CCA GTG AGC AGA GTG ACG-3'), was first applied to obtain it's 3' partial sequence. The SMART first-strand cDNA was synthesized to serve as a template for 5'RACE with the SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). Based on the 3' partial sequence, the anti-sense primer (5'-TCT CCA ATG ATA GAC GCA GGT ACA-3') was designed corresponding to the peptide fragment CTCVYHWR, a part of the newly discovered peptide sequence, and paired with the 5' nested universal primer A (5'-AAG CAG TGG TAT CAA CGC AGA GT-3'). PCR amplification was performed by using a 3'-RACE-PCR protocol composed of an initial denaturation step of 95 °C for 10 min, 30 cycles of 95 °C for 1 min, 42 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 7 min and a 5'-RACE-PCR protocol composed of an initial denaturation step of 94 °C for 10 min, 30 cycles of 95 °C for 30 s, 64 °C 10 s and 72 °C for 2 min and a final extension of 72 °C for 7 min. All PCR products were analyzed on a 2.5% (w/v) agarose gel and ligated into the pGEM-T vector (Promega, Madison, WI, USA). Sequencing was performed on the ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA). Data analysis was achieved using the Sequencing Analysis 2.1.2 and Sequence Navigator 1.0.1 (Applied Biosystems, Foster City, CA, USA) software.

2. 3. Phylogenetic Analysis

The αD-conopeptide sequences were aligned using Clustal W2.[13] Phylogenetic trees were constructed using the neighbour-joining (NJ)[15] and the maximum likelihood (ML)[16] methods. The reliability of the resulting topologies was evaluated by 1000 bootstrap replications. *C. achatinus* S-superfamily conotoxin Ac8.1 (ACA63847) was used as outgroup. Phylogenetic analysis of αD-conopeptide precursors or mature peptides was performed with the programs RAxML[16], Treecon[17] and MEGA 4.0[18].

3. Results

3. 1. Cloning of αD-conopeptide Precursor Sequences

αD-conopeptide precursor sequences were identified in venom glands of only four of the 27 *Conus* species

Figure 1. The cone snail species used in the present study (from left): *Conus betulinus, C. mustelinus, C. capitaneus, C. vexillum.*

Table 1. αD-conopeptide precursor amino acid sequences deduced from cDNA nucleotide , the mature peptide region is underlined, the AVV and EMM motif in the signal sequence are in bold. The prepro-region of *Conus capitaneus* was not completed.

C. mustelinus

MPKL**AVV**LLVLLILPLSYFDAAGGQVVQGDRRGNGLARYLQRGDRDVRECQVNTPGSKWGKCCMTRMCGTMCCARSGCTCVYHWRRGHGCSCPG

C. betulinus

MLKL**EMM**LVVLLILPLFYFDAAGGQVVQGDWRSDGLARYLQRGDRDVRECNINTPGSSWGKCCLTRMCGPMCCARSGCACVYHWRRGHGCSCPG

C. capitaneus

xxxxxxxxxxxxxxxxxxxxAAGGQVVQGDRRGNGLARYLQRGDREVQECQVDTPGSSWGKCCMTRMCGTMCCSRSVCTCVYHWRRGHGCSCPG

C. vexillum

MPKL**EMM**LLVLLIFPLSYFIAAGGQVVQVDRRGDGLAGYLQRGDRDVQDCQVSTPGSKWGRCCLNRVCGPMCCPASHCYCVYHRGRGHGCSC

investigated: *Conus betulinus, capitaneus, mustelinus* and *vexillum* (Figure 1). This adds another species, *C. betulinus*, to the six already known to contain these peptides (Table 1). The peptide from *C. vexillum* was found to be identical with Vx20.1 sequenced by Loughnan et al.,[5] those from *C. mustelinus* and *C. betulinus* were new for these species. But in contrast to the results of Loughnan et al.[5] screening of the cDNA from the venom gland of a *C. miles* specimen (from the Philippines) was negative for αD-conopeptide sequences.

The prepro-regions of the peptides consist of 44 (*C. betulinus*) and 45 (*C. mustelinus* and *C. vexillum*) amino acid residues and contain either the EMM- (*C. betulinus*) or AVV-motif (*C. mustelinus, vexillum*). The mature peptides have 47 (*C. vexillum*) and 49 (*C. mustelinus, C. betulinus*) amino acids including 10 cysteines, respectively (Table 1).

3. 2. Phylogenetic Analysis

Phylogenetic analysis of the αD-conopeptides revealed that they belong either to the AVV or EMM clades (Figure 2) as already reported by Loughnan et al.[5] The position of the *C. betulinus* αD-conopeptide is in the AVV clade, although it contains the EMM motif in the signal peptide. However, the *C. betulinus* αD-conopeptide possesses sequence motifs in the mature peptide that are typical for members of the AVV clade and are not present in EMM clade sequences, i.e. LTRM, ARSG and the PG motif at the C-terminus. The distribution of the αD-conopeptides in *Conus* species is very limited. They are present in three vermivorous *Conus* clades: in *Rhizoconus* (Clade XII) – *C. vexillum*, *C. mustelinus*, *C. miles*, *C. capitaneus*, and *C. rattus*, in Clade IX (*C. vitulinus*) and in Clade X (*C. betulinus*). These *Conus* clades were proposed by Espiritu et al.[19]

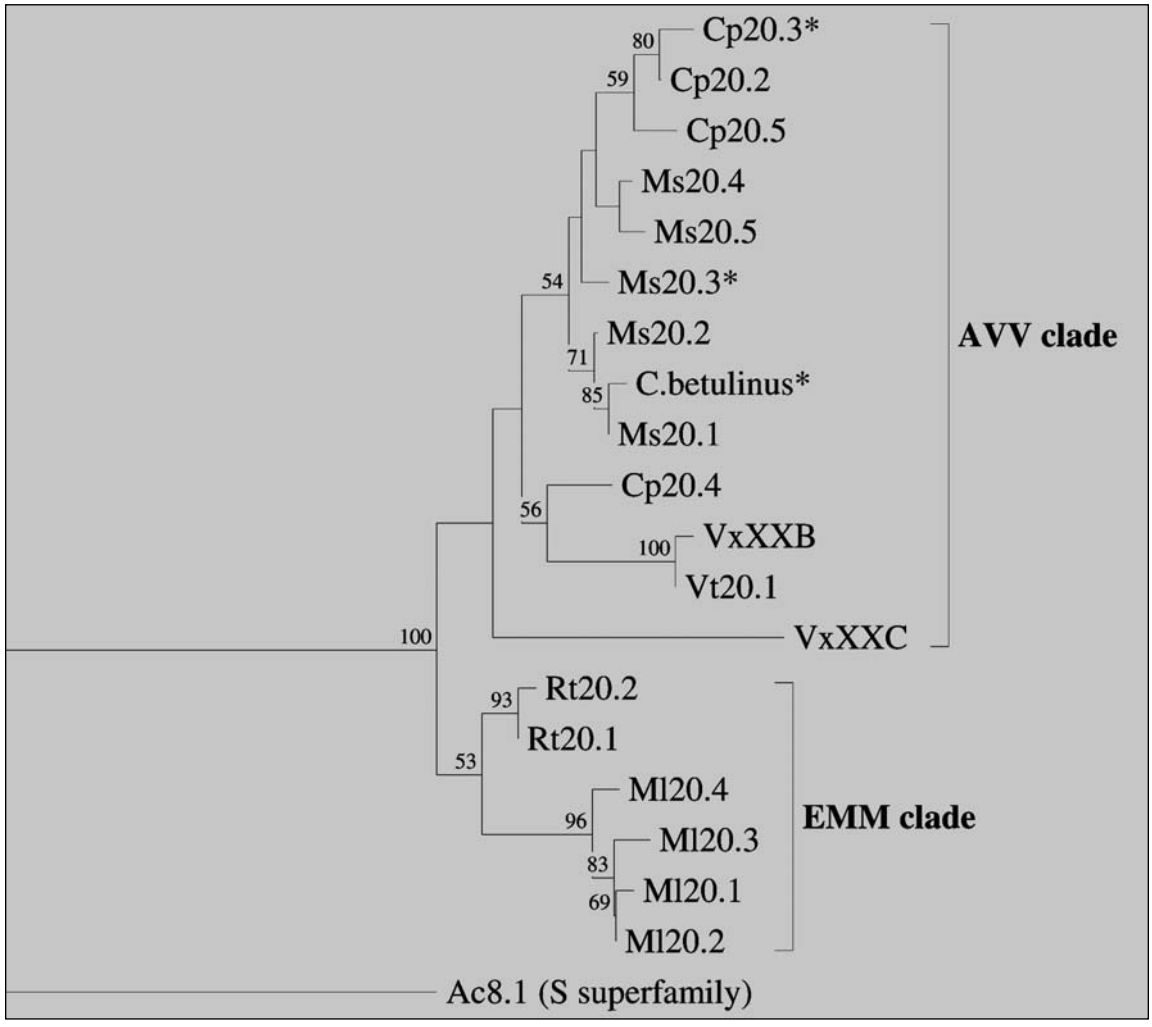

Figure 2. Phylogenetic tree of the αD-conopeptides. The rooted neighbour-joining (NJ) tree shows the evolutionary relationship among the currently available αD-conopeptides. Sequences that were obtained in this (*C. betulinus*) and in previous study[6] are marked with asterisks. The NJ tree represents the bootstrap consensus following 1000 replicates, nodes with confidence values greater than 50% are indicated. *C. achatinus* S-superfamily conotoxin Ac8.1 (ACA63847) was used as outgroup. The following abbreviations have been used by Loughnan et al.[5]: Cp – *Conus capitaneus*, Ml – *C. miles*, Ms – *C. mustelinus*, Rt – *C. rattus*, Vt – *C. vitulinus*, Vx – *C. vexillum*.

4. Discussion

αD-conopeptides have been identified by screening cDNA from venom glands or by isolation from the venom of *Conus capitaneus, miles, mustelinus, rattus, vitulinus, vexillum*[5,6,13] and *C. betulinus* (this paper). In several other species from the Philippines (Visaya Sea), Chesterfield Island (New Caledonia) and French Polynesia cDNA cloning did not indicate the presence of these peptides.

Loughnan et al.[5] observed two disctinct groups among the αD-conopeptide precursors containing either an EMM or AVV signal peptide motif. The new peptide from *C. betulinus* exhibits the EMM motif. Variable signal peptide sequences defining superfamily subgroups are not uncommon among other conopeptides such as in O-,[20,21] I-,[22,23] M-[24] and T-superfamilies.[25–27] Although the *C. betulinus* αD-conopeptide contains the EMM motif in the signal peptide, it is placed in the AVV clade, because it possesses typical sequence motifs in the mature peptide, which are not present in EMM clade sequences.

Although the *C. vitulinus* and the other αD-conopeptide possessing species diverged about 28 million years ago[28] their conopeptides are more similar to the AVV clade representatives than to the EMM clade. The αD-conopeptides from the EMM clade possessing species (*C. rattus* and *miles*) show 34 to 47% amino acid divergence in their mature peptides. While the majority of sequence replacements are scattered throughout the whole mature peptide, they contain in the inter-cysteine loops 2 and 4 two sequence motifs that are typical for EMM clade, the LNR(V/M) and P(A/E)SH, and they are also lacking the PG motif at the C-terminus. These sequence motifs could be responsible for possible changes in specificity and targeting. However, it should be noted that for the EMM clade sequences no biological activity or physiological targets are known yet.

It has been demonstrated that αD-conopeptides from the venom of *C. vexillum*,[5] *C. capitaneus* and *C. mustelinus*[6] specifically block mammalian neuronal nAChRs of the α7, α3β2 and α4β2 subtypes in nanomolar concentrations. Since all cone snail species which have been tested positive for αD-conopeptides are vermivorous, it would be interesting to investigate the affinity of these peptides to similar receptors in the nervous system of marine worms (polychaetes). The overall dominance of these peptides and the low abundance or even lack of small α-conopeptides in the venoms of these *Conus* species[6, 13] may suggest that αD-conopeptides are an adaptation to a specific type of prey, i.e. worms.

5. Acknowledgement

This work has been supported by a grant of the European Commission: CONCO, the cone snail genome project for health. Integrated Project ref. LSHB-CT-2007-037592 (http://www.conco.eu). We thank the Government of New Caledonia and French Polynesia for granting permissions, the French Navy and IRD-Nouméa for support as well as the members of the CONPOL 2007 and CONFIELD 2008 expedition for collecting and preparing the samples.

6. References

1. H. Terlau, B. M. Olivera, *Physiol. Rev.* **2004**, *84*, 41–68.
2. B. M. Olivera, R. W. Teichert, *Mol. Interv.* **2007**, *7*, 251–260.
3. H. R. Arias, M. P. Blanton, *Int. J. Biochem. Cell Biol.* **2000**, *32*, 1017–1028.
4. E. C. Jimenez, B. M. Olivera, R. W. Teichert, *Biochemistry* **2007**, *46*, 8717–8724.
5. M. Loughnan, A. Nicke, N. Lawrence, R. J. Lewis, *Biochemistry* **2009**, *48*, 3717–3729.
6. S. Kauferstein, Y. Kendel, A. Nicke, F. I. V. Coronas, L. D. Possani, P. Favreau, I. Križaj, C. Wunder, G. Kauert, D. Mebs, *Toxicon* **2009**, *54*, 295–301.
7. C. Peng, S. Tang, C. Pi, J. Liu, F. Wang, L. Wang, W. Zhou, A. Xu, *Peptides* **2006**, *27*, 2174–2181.
8. A. Nicke, S. Wonnacott, R. J. Lewis, *Eur. J. Biochem.* **2004**, *271*, 2305–2319.
9. R. Jacobsen, D. Yoshikami, M. Ellison, J. Martinez, W. R. Gray, G. E. Cartier, K. J. Shon, D. R. Groebe, S. N. Abramson, B. M. Olivera, J. M. McIntosh, *J. Biol. Chem.* **1997**, *272*, 22531–22537.
10. C. Hopkins, M. Grilley, C. Miller, K. J. Shon, L. J. Cruz, W. R. Gray, J. Dykert, J. Rivier, D. Yoshikami, B. M. Olivera, *J. Biol. Chem.* **1995**, *270*, 22361–22367.
11. R. W. Teichert, J. Rivier, J. Dykert, L. Cervini, J. Gulyas, G. Bulaj, M. Ellison, B. M. Olivera, *Toxicon* **2004**, *44*, 207–214.
12. R. W. Teichert, E. C. Jimenez, B. M. Olivera, *Biochemistry* **2005**, *44*, 7897–7902.
13. M. Loughnan, A. Nicke, A. Jones, C. I. Schroeder, S. T. Nevin, D. J. Adams, P. F. Alewood, R. J. Lewis, *J. Biol. Chem.* **2006**, *281*, 24745–24755.
14. M. A. Larkin, G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, D. G. Higgins, *Bioinformatics* **2007**, *23*, 2947–2948.
15. N. Saitou, M. Nei, *Mol. Biol. Evol.* **1987**, *4*, 406–425.
16. A. Stamatakis, *Bioinformatics* **2006**, *22*, 2688–2690.
17. Y. van de Peer, R. de Wachter, *Comput. Appl. Biosci.* **1997**, *13*, 227–230.
18. K. Tamura, J. Dudley, M. Nei, S. Kumar, *Mol. Biol. Evol.* **2007**, *24*, 1596–1599.
19. D. J. D. Espiritu, M. Watkins, V. Dia-Monje, G. E. Cartier, L. J. Cruz, B. M. Olivera, *Toxicon* **2001**, *39*, 1899–1916.
20. S. Kauferstein, C. Melaun, D. Mebs, *Peptides* **2005**, *26*, 361–367.
21. H. Jiang, C. Q. Xu., C. Z. Wang, C. X. Fran, T. T. Zhao, J. S. Chen, C. W. Chi, *Toxicon* **2006**, *47*, 425–436.

22. S. Kauferstein, I. Huys, U. Kuch, C. Melaun, J. Tytgat, D. Mebs, *Toxicon* **2004**, *44*, 539–548.
23. O. Buczek, D. Yoshikami, M. Watkins, G. Bulaj, E.C. Jimenez, B. M. Olivera, *FEBS J.* **2005**, *272*, 4178–4188.
24. Q. Wang, H. Jiang, Y.H. Han, D. D. Yuan, C. W. Chi, *Toxicon* **2008**, *51*, 813–822.
25. Y. H. Han, Q. Wang, H. Jiang, X. W. Miao, J. S. Chen, C. W. Chi, *Toxicon* **2005**, *45*, 481–487.
26. W. H. Chen, Y. H. Han, Q. Wang, X. W. Miao, L. Ou, X. X. Shao, *Acta Biochim. Biophys. Sinica* **2006**, *38*, 287–291.
27. C. Pi, J. Liu, C. Peng, X. Jiang, Y. Zhao, S. Tang, L. Wang, M. Dong, S. Chen, A. Xu, *Genomics* **2006**, *88*, 809–819.
28. S. T. Williams, T. F. Duda, *Evolution* **2008**, *62*, 1618–1634.

Povzetek

V strupih morskih polžev stožcev (*Conus* spp.) so našli številne biološko aktivne proteine in peptide, ki delujejo na ionske kanalčke in receptorje. Konopeptidi, ki delujejo na živčne nikotinske acetilholinske receptorje spadajo v različne konopeptidne naddružine. Mednje spadajo tudi nedavno odkriti αD-konopeptidi, ki tvorijo homodimere z 4749 aminokislinskimi ostanki. Analiza prisotnosti αD-konopeptidov v strupnih žlezah 27 različnih vrst stožcev (rod *Conus*) s pomočjo RT-PCR in cDNA kloniranja je potrdila njihovo prisotnost le pri štirih vrstah (*C. betulinus, C. capitaneus, C. mustelinus, C. vexillum*). Filogenetska analiza vseh trenutno dostopnih αD-konopeptidov je pokazala, da spadajo v dve skupini, ki se razlikujeta po prisotnosti AVV- ali EMM-motiva v zaporedju signalnega peptida.