Three-Dimensional Structure of the Mini-M Conotoxin mr3a[†]

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ABSTRACT: Conotoxin mr3a from the venom of *Conus marmoreus*, a novel peptide that induces rolling seizures in mice, has the peptide sequence GCCGSFACRFGCVOCCV, where O is *trans*-4-hydroxyproline, and the chain is cross-linked with disulfide bonds between Cys-2 and Cys-16, Cys-3 and Cys-12, and Cys-8 and Cys-15. The tertiary structure of mr3a was determined by 2D ^{1}H NMR in combination with a standard distance−geometry algorithm. The final set of 22 structures for the peptide had a mean global backbone RMS deviation of 0.53 ± 0.22 Å based on 51 NOE, 6 hydrogen bond, 6 ϕ dihedral angle, and 3 disulfide bond constraints. Conotoxin mr3a is the first example of the new mini-M branch of conopeptides in the M superfamily. Members of the maxi-M branch, whose structures are known, include the μ- and ψ-conotoxins, both of which share a common disulfide bond connectivity. Although mr3a has the same arrangement of Cys residues as the μ- and ψ-conotoxins, its disulfide connectivity is different. This gives mr3a a distinctive "triple-turn" backbone.

The venoms of predatory cone snails (*Conus*) are loaded with relatively small (10−40 amino acids), disulfide-rich peptides (conopeptides or conotoxins). It has been estimated that the venom duct of each *Conus* species produces 50−200 peptides that have evolved to immobilize prey consisting of fish, worms, or other snails (*1*, *2*). Thus, the snails appear to pursue a strategy that is biochemically analogous to that of a large pharmaceutical company by synthesizing a large series of pharmacological agents based on a relatively small number of rigid scaffolds.

Conus peptides typically have from two to five disulfide cross-links between cysteine residues found in characteristic arrangements of the amino acids within the primary sequence of the peptides. A significant fraction of conopeptides have three disulfide cross-links. Within this group four general Cys patterns are seen. The O superfamily (C−C−CC−C−C) includes ω- and κ-contoxins, which target voltage-gated calcium and potassium channels, respectively (*3*, *4*), and the δ- and μO-conotoxins, which inhibit voltage-gated sodium channel inactivation and conductance, respectively (*5*, *6*). The αA-conotoxins, which are competitive nicotinic antagonists (*7*), and the κA-conotoxins, which modulate the activity voltage-gated ion channels (*8*), have CC−C−C−C−C patterns. A third pattern (C−C−C−C−C−C) is seen in the P superfamily, which includes the spasmodic peptide that induces spasmodic and spastic behavior in mice (*9*). A fourth disulfide pattern (CC−C−C−CC) is found in a group of conotoxins that appear to be genetically related to and have been recently classified as the M superfamily (Figure 1) (*10*).

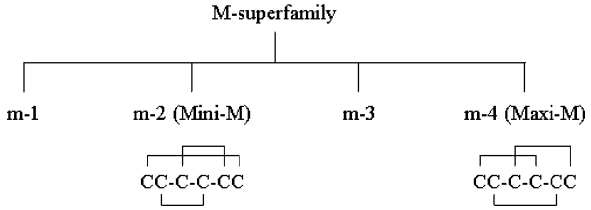

FIGURE 1: M superfamily of conotoxins. The four families in the M superfamily have CC−C−C−CC cysteine motifs. Disulfide bonding patterns are shown for the m-2 (mini-M) and m-4 (maxi-M) families.

Two different disulfide bonding patterns are seen in members of the M superfamily. The μ-, κM-, and ψ-conotoxins have Cys-1−Cys-4, Cys-2−Cys-5, and Cys-3−Cys-6 disulfide bonds. Although these peptides share common scaffolds, their biological activities are strikingly different: they block voltage-gated sodium (μ) and potassium (κM) channels (*11*) and acetylcholine receptors (ψ) (*12*, *13*).

Two distinctive peptides were recently reported for the mollusk-hunting conus snails *Conus textile* and *Conus marmoreus* (*10*). Included in this group are the "scratcher" peptides tx3c from *C. textile* (*13*)) and mr3a from *C. marmoreus*, which induce scratching behavior in mice (*10*). These small peptides (12−19 amino acids) have a unique Cys-1−Cys-6, Cys-2−Cys-4, and Cys-3−Cys-5 pattern of disulfide bonds and have been classified as the mini-M group within the superfamily.

The arrangement of disulfide cross-links largely dictates the shape of the scaffold in conotoxins. Three-dimensional structures of nicotinic acetylcholine receptor inhibiting ψ-conotoxins P_{IIIE} and P_{IIIF} from *Conus purpurascens* were recently reported (*14*, *15*). The backbone conformations of the two peptides are similar, although one of the disulfide loops in ψ-P_{IIIF} appears to be flexible. We now report a three-

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dimensional structure for conotoxin mr3a, the first member of the mini-M family to be characterized.

Conotoxin mr3a

MATERIALS AND METHODS

Preparation of mr3a. The mr3a peptide (*10*) was purified by preparative RP-HPLC. Cysteine residues were oxidized with glutathione, the resulting mixture of isomers was separated by RP-HPLC, and the appropriate fractions were combined. The sample was analyzed by HPLC, where it comigrated with the native conopeptide, and was active in the scratching assay (*10*).

NMR Experiments. NMR samples were prepared at a concentration of approximately 6.9 mM in either 100% D_2O (Cambridge Isotopes) or 9:1 (v/v) H_2O/D_2O with 0.01% TFA, pH 3.0. NMR spectra were acquired at 11.75 T on a Varian INOVA 500 NMR spectrometer at 26 °C. Proton DQF-COSY (*16*), NOESY (*17*), and TOCSY (*18*) spectra in D_2O and in H_2O were acquired with the transmitter set at 4.80 ppm and a spectral window of 6500 Hz, giving rise to FIDs of 4096 complex points in the ω_2 dimension. Suppression of the H_2O resonance was accomplished by transmitter presaturation. A series of NOESY spectra were acquired with mixing times of 400, 200, 150, 100, and 50 ms. TOCSY spectra under both solvent conditions were acquired with a mixing time of 200 ms. Spectra were processed on a Silicon Graphics Indigo 2 workstation using Varian VNMR software.

Restraint Set Generation. An initial survey of distance constraints was performed on a series of NOESY spectra acquired at mixing times of 400, 200, 150, 100, and 50 ms. Buildup curves were produced that demonstrated a leveling of the intensity of the nuclear Overhauser effect at mixing times over 200 ms. Visual inspection of the 200 ms NOESY spectrum identified 96 dipolar couplings that appeared on both sides of the diagonal and 85 that appeared on only one side of the diagonal. The off-diagonal resonances were classified as strong, medium, or weak on the basis of their relative intensities and set to distance constraints of 1.8−2.7, 1.8−3.3, and 1.8−5.5 Å, respectively (*19*). A set of 53 inter- and intraproton distance restraints representing unambiguously assigned dipolar couplings, known to be distance constraining, was generated from these data and used as input for DYANA (V. 1.4).

Six ϕ dihedral angles were determined on the basis of the $^3J_{NH_\alpha}$ coupling constants derived by visual analysis of a high-resolution 1D proton spectrum of conotoxin mr3a. ϕ angle constraints were set to $-120 \pm 40°$ for $^3J_{NH_\alpha}$ greater than 7.5 Hz (Cys-3, Ser-5, Val-13, Val-17) and to $-65 \pm 25°$ for $^3J_{NH_\alpha}$ less than 5 Hz (Phe-6, Ala-7) (*19*). For peaks that did not show a splitting pattern, the $^3J_{NH_\alpha}$ was derived from a measure of the line width at half the height of the signal.

Structure Calculations. One thousand random structures were generated by DYANA (V. 1.4) that fit the primary sequence and covalent and spatial requirements of mr3a. A total of 159 nonredundant distance constraints and six ϕ angle restraints were input for the molecular modeling protocol for the DYANA algorithm. A target function cutoff of seven was used to select structures that best fit the experimental distance and angle constraints. Eighty-eight structures met this criterion and were carried on to additional three rounds of optimization using target function cutoffs of 5, 3, and 1.5, respectively. The outcome was a set of 22 structures with a mean global RMS deviation of 0.53 ± 0.22 Å and a mean global heavy atom RMS deviation of 1.27 ± 0.30 Å.

Table 1: Proton Resonance Assignments (ppm) for mr3a

residue	HN	α	β	other
Gly1	9.10	4.16, 4.05		
Cys2	9.13	4.57	3.37, 2.84	
Cys3	8.62	4.87	3.19, 2.94	
Gly4	8.50	4.17, 3.91		
Ser5	8.86	4.56	3.31, 3.24	
Phe6	8.38	4.26	3.17, 3.11	2,4,6H, 7.32 3,5H, 7.37
Ala7	8.10	4.20	1.53	
Cys8	7.46	4.45	2.76, 2.55	
Arg9	7.53	4.01	1.64, 1.57	γ, 1.48, 1.32 δ, 3.01, 3.01 Nϵ, 7.03 NH1, 7.19 NH2, 6.67
Phe10	7.37	4.71	3.51, 2.76	2,4,6H, 7.16 3,5H, 7.01
Gly11	7.54	4.36, 3.74		
Cys12				
Val13	8.30	4.69	2.15	γ, 1.02, 0.94
Hyp14		4.76	2.35, 2.19	γ, 3.75, 3.52
Cys15	7.09			
Cys16	9.63	4.73	3.44, 3.23	
Val17	8.24	4.24	2.17	γ, 0.94, 0.94

RESULTS

Assignment of Resonances. The proton resonances for conotoxin mr3a were assigned by established methods (*20*). Fourteen of the seventeen spin systems were found in the "fingerprint" region of a 200 ms TOCSY spectrum (see the Supporting Information) and assigned to Gly-1, Cys-2, Cys-3, Gly-4, Ser-5, Phe-6, Ala-7, Cys-8, Arg-9, Phe-10, Gly-11, Val-13, Cys-16, and Val-17 (Table 1). The TOCSY assignments for Gly-1, Cys-2, Ser-5, Phe-6, Ala-7, Cys-8, Arg-9, Gly-11, Val-13, and Val-17 were verified in the fingerprint region of a DQF-COSY spectrum.

Sequential Assignments. The sequential assignments of amino acids in the primary sequence started with the unique residues Ala-7 and Arg-9. A NOESY "walk" toward the amino terminus identified the residues from Ala-7 to Cys-2 and toward the carboxy terminus from Cys-8 to Gly-11. Our previous assignments of the phenylalanines at positions 6 and 10 were confirmed during this process. Two of the glycine spin systems identified in the TOCSY spectrum were encountered in the NOESY walk at positions 4 and 11. The final glycine residue, Gly-1, was assigned to the spin pattern originating at 9.10 ppm by the process of elimination. The valine residues at positions 13 and 17 were assigned residues next. Val-17 was assigned to the spin system at 8.24 ppm on the basis of an NOE from the amide proton of Cys-16 to the β proton of Val-17. An NOE from the amide proton of Cys-15 to the β protons of Cys-16 led to the assignment of Cys-16 at 9.63 ppm and Cys-15 at 7.09 ppm. By the process of elimination, Val-13 was assigned to 8.30 ppm.

All of the side chain protons were assigned except for Cys-12 and Cys-15. The amide proton of Cys-15 was assigned to a peak at 7.09 ppm on the basis of an observable NOE to the β protons in Cys-16 at 3.44 and 3.23 ppm seen in the fingerprint region of the 400 ms NOESY spectrum. However, the rest of the spin system could not be identified. At this point the only residue not accounted for was Cys-12. The two possible locations for the Cys-12 spin system are the aromatic region or the regions at 9.10 and 9.13 ppm occupied by Gly-1 and Cys-2. Although other approaches such as labeling with a heavy isotope (^{13}C or ^{15}N) could be used, the assignment of Cys-12 was not deemed necessary for structure calculations because it is restrained by the disulfide bond to Cys-3. Often the constraints for cysteine residues in conotoxins, aside from the disulfide bond, are minimal because of poor NOESY data. However, the NOESY data acquired at 26 °C for conotoxin mr3a showed a large number of NOEs that suggested the structure of the peptide was sufficiently constrained for distance−geometry calculations.

Evaluation of Structure. Conotoxin mr3a provided relatively few complications during the sequential assignment of residues, the extraction of distance and angle information, and the determination of three-dimensional structure. The lack of proton chemical shift data and restraint assignments for Cys-12 and Cys-15 was not deemed critical for preliminary structure minimization. The constraints for structure elucidation were determined from a survey of NMR data by the traditional visual analysis method developed by Wüthrich (*20*). The procedure involved an interpretation of the 96 dipolar couplings that appeared on both sides of the diagonal in the 200 ms NOESY spectrum. Forty-one of the observed off-diagonal resonances could be unambiguously assigned and were used as distance constraints. The 6 ϕ angle constraints and 3 disulfide bonds from Cys-2 to Cys-16, from Cys-3 to Cys-12, and from Cys-8 to Cys-15 were added to the 41 distance constraints for preliminary structure determination. A set of 20 structures was generated with a mean global RMS deviation of 2.04 Å using the DYANA (V. 1.4) software package. The lowest energy structure was then displayed, and the ambiguous NOESY signals were evaluated compared to those of the partially minimized structure. Twelve distance constraints were added on the basis of this analysis, and the minimization process was repeated to generate a set of 13 structures with a mean global RMS deviation of 0.76 Å.

At this stage of the structure elucidation process, the DYANA program was prompted to provide hydrogen bond information during the minimization. Deuterium exchange studies indicated that hydrogen bonds might exist between the amide protons of Cys-2, Ser-5, Ala-7, Cys-8, and Val-13 and nearby oxygen or nitrogen atoms. The resonances of amide protons in these residues were not diminished after 3 h in D_2O at 26 °C in a 1D proton time course experiment. DYANA provided hydrogen bond acceptor oxygen and nitrogen atoms for each of the amide protons from these five residues. The amide proton of Cys-2 was estimated to be within hydrogen-bonding distance of oxygen atoms in Cys-8 and Arg-9 ($\leq$3 Å). The amide proton of Ala-7 was found to be within an acceptable hydrogen bond distance to the N-terminal nitrogen atom of Gly-1. The N-terminal nitrogen is expected to be fully protonated at pH 3, and a hydrogen bond to the nitrogen atom is not appropriate. The hydrogen bonds for Cys-2, Ser-5, Cys-8, and Val-13 were used as constraints, and structures were generated again in an attempt to locate a hydrogen bond between the amide proton of Ala-7 and an acceptor atom. At this point the resistance of the amide proton to deuterium exchange remains unclear. It is possible that the amide proton is shielded from bulk water, but this would be unusual for a small peptide. Thus, 10 upper and 10 lower distance constraints were added for the hydrogen bond interactions, and another round of minimization was performed as described by Volkman et al. (*21*). The result was a final set of 22 structures with a mean global backbone RMS deviation of 0.53 $\pm$ 0.22 Å and a mean global heavy atom RMS deviation of 1.28 $\pm$ 0.31 Å. The constraints per residue used to generate the final set of structures are summarized in Table 2 and Figure 2.

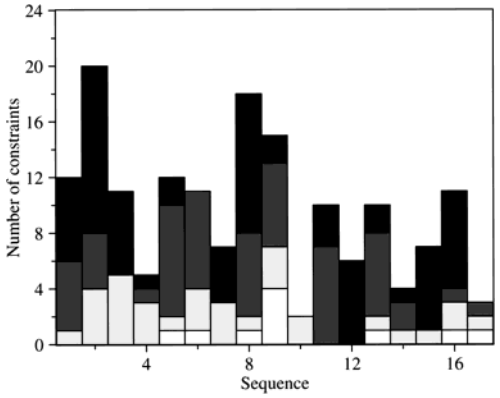

FIGURE 2: A plot of the distribution of distance constraints as a function of residue number used for the structure determination of conotoxin mr3a. The white areas represent intraresidual constraints, light gray areas symbolize sequential constraints, dark gray areas show medium-range NOEs ($d_{ij} \leq 5$ Å), and black areas depict long-range NOEs ($d_{ij} \geq 5$ Å).

Table 2: Restraints Used To Determine the Structure of mr3a

distance restraints		dihedral restraints	
intraresidue	10	ϕ	6
sequential	32	hydrogen bond	6
medium	49	total	12
long	68		
total	159		

Description of Structures. The overall RMS deviation reported for the final set of 22 structures (0.53 $\pm$ 0.22 Å) is influenced by the disorder in the C-terminal valine. When Val-17 is removed and the molecule is minimized considering the first 16 residues only, the mean global backbone RMS deviation drops from 0.53 to 0.38 Å. The final structure serves as a template for mini-M peptides within the M superfamily. Figure 3 shows an overlay of the backbone (N, CA, C, and O) atoms, for the 22 structures of mr3a. The N-terminal portion of the molecule is very well resolved, while the C-terminal valine is poorly resolved.

Conotoxin mr3a folds into a tight globular structure containing three turns defined by residues Gly-1 to Ser-5, Ser-5 to Phe-10, and Phe-10 to Cys-15 (Figure 4). The distance between the amide proton of Ser-5 and the carbonyl oxygen of Cys-2 in the first loop is 2.30 Å, consistent with a hydrogen bond between the two atoms. This interaction is characteristic of a type I β turn (*22*) with a cysteine (Cys-2) residue at position *i* and a glycine residue (Gly-4) at position $i + 2$. The glycine residue is required to accommodate the

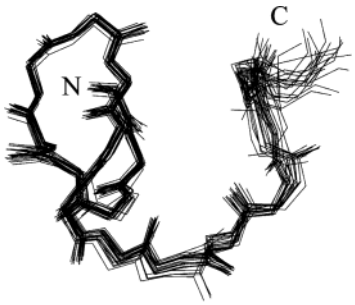

FIGURE 3: An overlay of the backbone atoms for the 22 structures generated by DYANA representing mr3a. The C-terminal valine is observed to be in a poorly resolved region of the molecule, whereas the N-terminal glycine is in a well-resolved portion of the molecule.

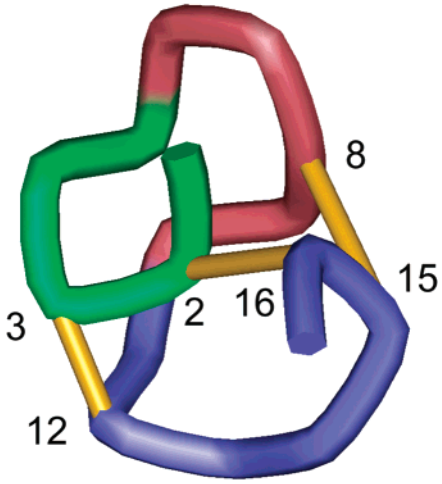

FIGURE 4: Backbone structure of mr3a. Turn 1 between Gly[1] and Ser-5 (shown in green) is reinforced by a hydrogen bond between Cys-2 and Ser-5. Turn 2 between Ser-5 and Phe-10 (shown in red) is reinforced by a hydrogen bond between Cys-8 and Phe-6. Turn 3 between Cys-8 and Cys-16 (shown in blue) is reinforced by a hydrogen bond between Val-13 and Gly-11.

necessary angle constraints of the turn. The second turn in the region between Ser-5 and Phe-10 is apparently stabilized by a hydrogen bond between the carbonyl oxygen of Phe-6 and the amide proton of Cys-8. These two atoms are separated by a distance of 2.37 Å. This motif is characteristic of a classical γ turn with a hydrogen bond between the carbonyl oxygen of residue *i* and the amide proton of residue $i + 2$ (*22*). The third turn is constrained by the disulfide bonds between Cys-8 and Cys-15 and Cys-2 and Cys-16. There is also a hydrogen bond between the amide proton of Val-13 and the carbonyl oxygen of Gly-11. The structure in this region of the backbone is also characteristic of a γ turn. This motif is defined by several NOEs, including those between the amide and α protons of Gly-11 and the β, δ, and γ protons of Arg-9 and between the amide and β protons of Val-13 and the δ protons of Arg-9. The 4-hydroxyproline residue in position 14 was modeled by a proline residue during structure calculations. The NMR data indicated very little interaction between the δ protons of Pro-14 and the rest of the molecule. The Pro-14 ring points away from the rest of the molecule such that a hydroxyl group in the δ position could enhance the hydrophilic nature of the molecule.

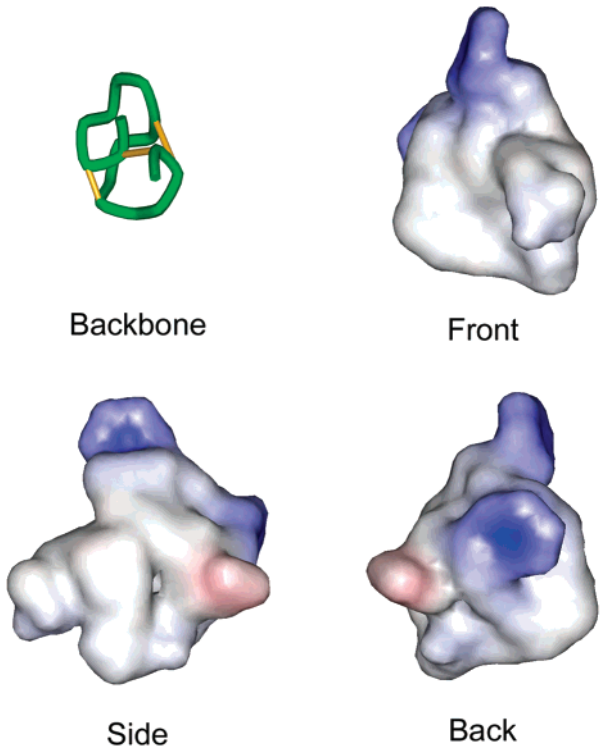

FIGURE 5: Three-dimensional structures of mr3a. Shown are the backbone structure along with front, side, and back views of the surface of the peptide. Blue regions are hydrophobic, and red regions are hydrophilic.

DISCUSSION

The 17 amino acid *C. marmoreus* peptide mr3a is a compact globular peptide with a well-defined tertiary structure (Figure 5). The peptide has the same arrangement of Cys residues as the μ- and ψ-conotoxins (CC−C−C−CC). mr3a is closely related to tx3c from *C. textile* and belongs to a group of small conopeptides that have a Cys-1−Cys-6, Cys-2−Cys-4, and Cys-3−Cys-5 pattern of disulfide bonds. This pattern is different from the Cys-1−Cys-4, Cys-2−Cys-5, and Cys-3−Cys-6 arrangement seen in the μ-conotoxins (*23*) and ψ-conotoxins (*14*, *15*). As a consequence, the backbone conformation of mr3a is different from those reported for μ- and ψ-conotoxins. We refer to the mr3a fold as a "triple-turn" scaffold. In effect, the N- and C-terminal ends of the molecule are covalently joined by the Cys-1−Cys-6 disulfide bond. The Cys-2−Cys-4 and Cys-3−Cys-5 bonds stitch the middle of the molecule together to produce the characteristic triple-turn motif.

The backbone fold of mr3a is different from those of the maxi-M conotoxins φ-PIIIE and μ-GIIIA as a result of the different disulfide linkages in the CC−C−C−CC pattern. Thus, differences in disulfide connectivity are used to generate different folds for the backbone scaffold for common arrangements of cysteine residues within the peptide chain. An analogous situation was recently described for the T superfamily, where two different branches exhibit different disulfide connectivity (*24*). Although, the patterns of disulfide bonds for other peptides in the mini-M family have not been determined, their close genetic relationship and their primary amino acid sequences suggest that they have the same connectivity found in mr3a and tx3c.

The maxi-M peptides μ-GIIIA, μ-GIIIB, ψ-PIIIE, and ψ-PIIIF have similar structures. There is a high degree of

similarity in the backbone conformations and sequence homology between ψ-PIIIE and ψ-PIIIF (*14*, *15*). Both peptides have the shape of a lens with the N-terminus on the convex face and the C-terminus on the concave face. The overall shape of the ψ-conotoxins, when viewed from the front or back, is triangular. The triple-turn conformation in conopeptide mr3a produces the overall shape of a "plucked chicken" when viewed from the front (Figure 5).

The maxi-M μ- and ψ-conotoxins are found mostly in fish-hunting *Conus* species, whereas the mini-M peptides are in the venom of mollusk-hunting and worm-hunting cone snails. Peptides with the mini-M sequence motif have not been found in fish-hunting *Conus* venoms. Although the molecular target of peptide mr3a has yet to be determined, the strikingly different symptomatology induced by this peptide compared to the μ- and ψ-conotoxins deserves comment. μ-Conotoxins are channel blockers targeted to voltage-gated sodium channels, and ψ-conotoxins are noncompetitive antagonists of the nicotinic acetylcholine receptors. The peptides in these two families that have been characterized so far are paralytic toxins which interfere with neuromuscular transmission. In contrast, mr3a causes excitatory effects, including hyperactivity, barrel rolling, and convulsions. It seems unlikely that the peptide targets either voltage-gated sodium channels or nicotinic acetylcholine receptors. Thus, they may represent a novel pharmacological family of conotoxins.

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SUPPORTING INFORMATION AVAILABLE

Fingerprint region of a 200 ms TOCSY spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Olivera, B. M. (1997) *Mol. Biol. Cell 8*, 2101−2109.
2. Olivera, B. M., Walker, C., Cartier, G. E., Hooper, D., Santos, A. D., Schoenfeld, R., Shetty, R., Watkins, M., Bandyopadhyay, P., and Hillyard, D. R. (1999) *Ann. N. Y. Acad. Sci. 870*, 223−237.
3. Olivera, B. M., McIntosh, J. M., Cruz, L. S., Luque, F. A., and Gray, W. R. (1984) *Biochemistry 23*, 5087−5090.
4. Terlau, H., Shon, K. J., Grilley, M., Stocker, M., Stühmer, W., and Olivera, B. M. (1996) *Nature 381*, 148−151.
5. Shon, K., Hasson, A. Spira, M. E., Cruz, L. J., Gray, W. R., and Olivera, B. M. (1994) *Biochemistry 33*, 11420−11425.
6. McIntosh, J. M., Hasson, A., Spira, M. E., Gray, W. R., Li, W., Marsh, M., Hillyard, D. R., and Olivera, B. M. (1995) *J. Biol. Chem. 270*, 16796−16802.
7. Jacobsen, R., Yoshikami, D., Ellison, M., Martinez, J., Gray, W. R., Cartier, G. E., Shon, K. J., Groebe, D. R., Abramson, S. N., Olivera, B. M. and McIntosh, J. M. (1997) *J. Biol. Chem. 272*, 22531−22537.
8. Craig, A. G., Zafaralla, G., Cruz, L. J., Santos, A. D., Hillyard, D. R., Dykert, J., Rivier, J. E., Gray, W. R., Imperial, J., DeLaCruz, R. G., Sporning, A., Terlau, H., West, P. J., Yoshikami, D., and Olivera, B. M. (1998) *Biochemistry 37*, 16019−16025.
9. Lirazon, M. B., Hooper, D., Corpuz, G. P., Ramilo, C. A., Bandyopadhyay, P., Cruz, L. J., and Olivera, B. M. (2000) *Biochemistry 39*, 1583−1588.
10. Corpuz, G. P., Jacobsen, R., Jimenez, E. C., Watkins, M., Walker, C., Colledge, C., Garrett, J. E., McDougal, O. M., Li, W., Gray, W. R., Hillyard, D. R., Rivier, J. E., McIntosh, J. M., Cruz, L. J., and Olivera, B. M. (2003) *Biochemistry* (submitted for publication).
11. Ferber, M., Sporning, A., Jeserich, G., DeLaCruz, R., Watkins, M., Olivera, B. M., and Terlau, H. (2002) *J. Biol. Chem. 278*, 2177−2183.
12. Jacobsen, Cartier, G. E., Hopkins, C., Gray, W. R., Watkins, M., Hillyard, D. R., Rivier, J., Torres, J., Yoshikami, D., and Olivera, B. M. (1997) *Biochemistry 36*, 9581−9587.
13. Olivera, B. M., Rivier, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., Mena, E. E., Woodward, S. R., Hillyard, D. R., and Cruz, L. J. (1990) *Science 249*, 257−263.
14. Van Wagoner, R. M., and Ireland, C. M. (2003) *Biochemistry 42*, 6347−6352.
15. Van Wagoner, R. M., Jacobsen, R. B., Olivera, B. M., and Ireland, C. M. (2003) *Biochemistry 42*, 6353−6362.
16. Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479−485.
17. Jeener, J., Meier, B. H., Bachmann, P., and Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546−4553.
18. Braunschweiler, L., and Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521−528.
19. Bax, A. (1989) *Annu. Rev. Biochem. 58*, 223−256.
20. Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
21. Volkman, B. F., Nohaile, M. J., Amy, N. K., Kustu, S., and Wemmer, D. E. (1995) *Biochemistry 34*, 1413−1424.
22. Rose, G. D., Gierasch, L. M., and Smith, J. A. (1985) *Adv. Protein Chem. 37*, 1−109.
23. Hill, J. M., Alewood, P. F., and Craik, D. J. (1996) *Biochemistry 35*, 8824−8835.
24. Steel. B., Cruz, L. J., Hooper, D., Shetty, R., DeLaCruz, R., Cruzt, L., Nielsen, J. S., Zhou, L. M., Bandyopadhyay, P., Craig, A. G., and Olivera, B. M. (1999) *J. Biol. Chem. 274*, 30664−30671.

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