Inhibition of the Norepinephrine Transporter by the Venom Peptide χ-MrIA

SITE OF ACTION, Na⁺ DEPENDENCE, AND STRUCTURE-ACTIVITY RELATIONSHIP

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χ-Conopeptide MrIA (χ-MrIA) is a 13-residue peptide contained in the venom of the predatory marine snail Conus marmoreus that has been found to inhibit the norepinephrine transporter (NET). We investigated whether χ-MrIA targeted the other members of the monoamine transporter family and found no effect of the peptide (100 μM) on the activity of the dopamine transporter and the serotonin transporter, indicating a high specificity of action. The binding of the NET inhibitors, [3H]nisoxetine and [3H]mazindol, to the expressed rat and human NET was inhibited by χ-MrIA with the conopeptid displaying a slight preference toward the rat isoform. For both radioligands, saturation binding studies showed that the inhibition by χ-MrIA was competitive in nature. It has previously been demonstrated that χ-MrIA does not compete with norepinephrine, unlike classically described NET inhibitors such as nisoxetine and mazindol that do. This pattern of behavior implies that the binding site for χ-MrIA on the NET overlaps the antidepressant binding site and is wholly distinct from the substrate binding site. The inhibitory effect of χ-MrIA was found to be dependent on Na⁺ with the conopeptid becoming a less effective blocker of [3H]norepinephrine by the NET under the conditions of reduced extracellular Na⁺. In this respect, χ-MrIA is similar to the antidepressant inhibitors of the NET. The structure-activity relationship of χ-MrIA was investigated by alanine scanning. Four residues in the first cysteine-bracketed loop of χ-MrIA and a His in loop 2 played a dominant role in the interaction between χ-MrIA and the NET. Hα chemical shift comparisons indicated that side-chain interactions at these key positions were structurally perturbed by the replacement of Gly-6. From these data, we present a model of the structure of χ-MrIA that shows the relative orientation of the key binding residues. This model provides a new molecular caliper for probing the structure of the NET.

Because of its poor lipid solubility and degree of ionization at physiological pH, norepinephrine crosses cell membranes poorly by diffusion (1) and so relies on the operation of the norepinephrine transporter (NET)3 for uptake into cells. Clearance by this integral membrane protein constitutes the major mechanism for the termination of action of this neurotransmitter at noradrenergic synapses (2), and disturbances in the functioning of the NET are associated with pathological states including depression (3), congestive heart failure (4), and orthostatic intolerance, and tachycardia (5). Known inhibitors of the NET include antidepressants (e.g. desipramine and nisoxetine), the appetite suppressant mazindol, and the abused drug cocaine (for review, see Ref. 6). The NET, together with the dopamine transporter (DAT) and the serotonin transporter (SERT), forms a family of Na⁺- and Cl⁻-dependent monoamine transporters.

A novel peptidic NET inhibitor, χ-MrIA, has been identified in cone snail venom (7). Cone snails use a venom containing a mixture of bioactive peptides (“conopeptides”) to capture their prey, and these are known to target an array of voltage-sensitive ion channels, ligand-gated ion channels, and G protein-coupled receptors (for review, see Ref. 8). Intrathecal injection of χ-MrIA has been found to be analgesic in hot plate and neuropathic pain models (9, 10). The inhibition of [3H]norepinephrine uptake by the NET caused by χ-MrIA was found to be non-competitive, reducing the maximum rate of transport and not affecting the affinity of the transporter for substrate (7). The non-competitive mode of action of χ-MrIA distinguishes it from the majority of the classically described inhibitors of the NET that act in a competitive fashion. In this study, we explored the interaction of χ-MrIA with the monoamine transporters to gain an insight into the selectivity, Na⁺ dependence, site of action, and structure-activity relationship of the conopeptide.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—χ-MrIA and the singly substituted analogs, [N1A]MrIA, [G2A]MrIA, [V3A]MrIA, [G6A]MrIA, [K8A]MrIA, [L9A]MrIA, [H11A]MrIA, [Y7F]MrIA, and [K8R]MrIA, were synthesized. The chain assembly of the peptides was performed on a manual shaker system using HBTU activation protocols (11) to couple the Fmoc-protected amino acid to the resin. The Fmoc-protecting group was removed using 50% piperidine in dimethylformamide (12) before purification on RP-HPLC. The purity of the peptides was determined by mass spectrometry. The atomic coordinates and structure factors (code 1IEO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: NET, norepinephrine transporter; DAT, dopamine transporter; SERT, serotonin transporter; 5-HT, 5-hydroxytryptamine (serotonin); NOESY, Nuclear Overhauser enhancement spectroscopy; TOCSY, total correlated spectroscopy; HBTU, N,N′-diisopropylcarbodiimide-benzotriazol-1-yl)-uronium hexafluorophosphate; Fmoc, N-9-fluorenylmethoxycarbonyl; ANOVA, analysis of variance.
was deprotected and cleaved from the resin by stirring at room temperature in trifluoroacetic acid:H₂O:trisopropylsilane:ethanedithiol (90:5:5:4) for 2–3 h. Cold diethyl ether was then added to the mixture, and the peptide precipitated out. The precipitate was collected by centrifugation and subsequently washed with further cold diethyl ether to remove scavengers. The final product was dissolved in 50% aqueous acetonitrile and lyophilized to yield a fluffy white solid. The crude, reduced peptide was examined by reverse phase high performance liquid chromatography for purity, and the correct molecular weight was confirmed by electrospray mass spectrometry. Pure, reduced peptides were oxidized, and the major peak was purified to >95% purity and characterized by high performance liquid chromatography prior to further use.

**Cellular Uptake of [³H]Monoamines—** COS-1 cells (ATCC, Manassas, VA) were grown in 24-well plates (Falcon, BC Biosciences) containing Dulbecco’s modified Eagle medium (Invitrogen) and 10% fetal bovine serum (Invitrogen) at 37 °C in 5% CO₂. Upon reaching ~85% confluency, the cells were transiently transfected with plasmid DNA encoding the human NET (13), the rat NET (14), the human DAT (15), or the human SERT (16). Transfections were performed using LipofectAMINE 2000 reagent (Invitrogen) following the manufacturer’s protocol using 800 ng of DNA/well. Assays measuring the cellular accumulation of the transporters’ respective [³H]monoamine substrates were performed 24 h after transfection at room temperature in duplicate. The culture medium was removed, and the cells were washed three times with 500 μL of warm phosphate-buffered saline containing 125 mM NaCl, 4.8 mM KCl, 1.5 mM MgSO₄, 1.2 mM KH₂PO₄, 1.3 mM CaCl₂, 25 mM HEPES, 5.55 mM d(+)-glucose, 1.02 mM ascorbic acid, 10 μM U-0521 (to inhibit catechol-O-methyl transferase), and 100 μM pargyline (to inhibit monoamine oxidase), pH 7.4. In experiments examining the Na⁺ dependence of the NET inhibitors, the concentration of NaCl used in the transport buffer ranged from 25 to 125 mM with appropriate concentrations of LiCl added to retain equal osmolality. Inhibitor drugs were preincubated with the cells for 15 min before the addition of 100 nM [³H]monoamine substrate (supplemented with unlabeled substrate as required). The final volume was 250 μL. Non specific uptake of [³H]norepinephrine by NET-transfected cells was defined by the accumulation occurring in the presence of 100 μM desipramine, or [³H]mazindol (10 μM) and [³H]nisoxetine (10 μM). Nonspecific uptake of [³H]nisoxetine was not significantly altered (Fig. 1B). Transfected cells were then rapidly removed, and the cells were washed three times with 1 ml of ice-cold phosphate-buffered saline. The cells were then lysed with 0.1% Triton X-100 in 10 mM Tris-HCl, pH 7.5, for 60 min at room temperature with gentle shaking. The level of radioactivity of the cell lysate was determined by liquid scintillation counting.

**Membrane Preparation—** COS-7 cells (ECACC, Salisbury, Wiltshire, United Kingdom) were grown in 150-mm dishes and transiently transfected with 15 μg of plasmid DNA encoding the rat NET using the same method described for the uptake experiments. Membranes were prepared from cells 48 h after transfection for use in radioligand binding experiments. After washing the cells with warm phosphate-buffered saline, ice-cold TEM buffer (10 mM Tris-HCl, 1.4 mM EGTA, 12.5 mM MgCl₂, pH 7.5) was added and the cells were scraped from the dish. Cells were then homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and centrifuged at 1000 × g for 5 min at 4 °C to remove cellular debris and nuclei. The pellet of radiolabeled cells was determined by liquid scintillation counting.

**Statistics and Data Analysis**—All data are expressed as means ± S.E. of results obtained from 2 to 5 separate experiments. Student’s two-tailed t test or, where appropriate, ANOVA with post hoc shift tests performed by the Tukey method was used to evaluate the statistical significance of differences between groups. Values of p < 0.05 were considered significant. Curve fitting of concentration-response curves and radioligand binding data was performed by non-linear regression using individual data points with Prism 3.0 software for Macintosh (GraphPad, San Diego, CA). The equation of Cheng and Prusoff (24) was used to convert IC₅₀ values to Kᵥ values.

**RESULTS**

**Effect of χ-MrIA on the Cellular Uptake of [³H]Monoamines—** COS-1 cells transfected with either the rat or human NET readily accumulated [³H]norepinephrine, and nonspecific uptake of [³H]norepinephrine was <2.5% of the total uptake. As shown in Fig. 1, the uptake of [³H]norepinephrine via the rat and human NET was sensitive to inhibition by χ-MrIA with pIC₅₀ values of 6.21 ± 0.02 (rat; n = 3) and 5.90 ± 0.03 (human; n = 5). χ-MrIA acted as a full inhibitor of the NET of both species. For DAT- and SERT-transfected cells, nonspecific uptake represented <6% of the total [³H]norepinephrine accumulation. In the presence of χ-MrIA (100 μM), the rate of uptake of [³H]dopamine by the human DAT and [³H]serotonin by the human SERT was not significantly altered (Fig. 1B).
Effect of χ-MrIA on the Binding of Classical NET Inhibitors—χ-MrIA inhibited the binding of \([3H]\)nisoxetine to the membranes of cells expressing the rat and human NET (Fig. 2A). The IC\(_{50}\) for inhibition was 500 nM (pK\(_I\) = 6.6 ± 0.05) at the rat NET and 1.7 µM (pK\(_I\) = 6.0 ± 0.04) at the human NET. \([3H]\)Mazindol binding to the expressed transporters was also sensitive to χ-MrIA (Fig. 2B). χ-MrIA inhibited binding with an IC\(_{50}\) of 1.9 µM (pK\(_I\) = 6.4 ± 0.03) at the rat NET and 4.0 µM (pK\(_I\) = 5.9 ± 0.04) at the human NET. Nonspecific binding represented ~3% of the total binding in all of the experiments, and χ-MrIA acted as a full inhibitor of \([3H]\)nisoxetine and \([3H]\)mazindol binding.

Saturation analysis was used to characterize the nature of the inhibition caused by χ-MrIA (Fig. 3). In the absence of χ-MrIA, \([3H]\)nisoxetine bound to rat NET membranes with a K\(_d\) of 4.2 ± 0.5 nM and a B\(_{max}\) of 42 ± 1.6 pmol/mg protein. The K\(_d\) was increased to 21 ± 3.6 nM in the presence of 2 µM χ-MrIA with no significant change in the value of the B\(_{max}\) (45 ± 4.2 pmol/mg protein). For \([3H]\)mazindol binding, the K\(_d\) was 1.0 ± 0.1 nM and the B\(_{max}\) was 40 ± 0.3 pmol/mg protein in control experiments. In the presence of χ-MrIA (20 µM), the K\(_d\) (35 ± 1.5 nM) but not the B\(_{max}\) (41 ± 0.6 pmol/mg protein) was significantly altered. χ-MrIA (10\(^{-6}\) M) did not affect the dissociation rate of \([3H]\)nisoxetine from the expressed rat NET (data not shown).

Desipramine, nisoxetine, and χ-MrIA reduced the binding of \([3H]\)nisoxetine to rat brain homogenates in a concentration-dependent manner (Fig. 4). F tests comparing the fit of the binding data to a model of one-site competition, two-site competition, or a sigmoidal curve with a variable slope indicated that the simpler one-site competition model was preferred and more complicated models did not significantly improve the fit (p > 0.2 for each of the comparisons). The IC\(_{50}\) values for the inhibition were 1.1 nM (pIC\(_{50}\) = 8.9 ± 0.06) for desipramine, 6.2 nM (pIC\(_{50}\) = 8.2 ± 0.07) for nisoxetine, and 5.7 µM (pIC\(_{50}\) = 5.2 ± 0.22) for χ-MrIA. While desipramine and unlabeled nisoxetine inhibited the \([3H]\)nisoxetine binding to the same extent (non-specific binding of ~43%), the estimated maximum extent of inhibition produced by χ-MrIA was significantly less (p <
Structural Effects of Alanine Substitutions —

0.001) with ~32% of the nisoxetine- and desipramine-sensitive binding found to be insensitive to χ-MrIA.

Sodium dependence of NET inhibition —The rate of uptake of [3H]norepinephrine by cells transfected with the human NET slowed substantially as the concentration of Na⁺ in the transport buffer was reduced. At the lowest Na⁺ concentration examined (25 mM), the rate of [3H]norepinephrine accumulation was approximately 50% of that observed at 125 mM Na⁺ (data not shown). Concentrations of desipramine and χ-MrIA that inhibited transport by 50% in assays where the buffer contained 125 mM Na⁺ (4.05 μM and 1.26 μM, respectively) were found to inhibit progressively a smaller proportion of the uptake in buffer containing less Na⁺ (Fig. 5).

Effect of Residue Replacement on the Potency of χ-MrIA —

Nine analogs of χ-MrIA in which the non-cysteine residues were systematically replaced with alanine were assayed for inhibition of [3H]nisoxetine binding to the expressed human NET, and their potency was compared (Fig. 6). The analogs with substitutions at N-terminal residues outside of the cysteine-bracketed loops (N1A MrIA, G2A MrIA, and V3A MrIA) displayed no significant change in potency compared with χ-MrIA. The replacement of any of the residues located in the first cysteine-bracketed loop, in contrast, had a severe impact on potency. No inhibition was observed with these analogs ([G6A] MrIA, [Y7A] MrIA, [K8A] MrIA, and [L9A] MrIA) at 100 μM, the highest concentration tested. Assuming that the Hill slope parameter for their inhibition remains unchanged compared with χ-MrIA, the IC₅₀ concentrations of these peptides will be at least an order of magnitude greater still, yielding a conservative estimate of 10⁻³ M.

Alanine substitution of the first residue of the second cysteine-bracketed loop (analog H11A MrIA) caused a ~60-fold reduction in potency. Replacement of the other residue in this loop (analog O12A MrIA) did not have a significant effect on potency. Two further analogs were assayed to investigate the effect of replacement with residues other than alanine at positions 7 and 8. The potency of [Y7F] MrIA was ~3.8-fold lower (pIC₅₀ = 5.2 ± 0.08) than χ-MrIA, and the potency of [K8R] MrIA was ~6.8-fold lower (pIC₅₀ = 4.9 ± 0.10) than χ-MrIA.

Structural Effects of Alanine Substitutions —

Secondary chemical shifts, i.e. Hα chemical shifts compared with random coil values (25), are a sensitive measure of backbone conformation (26–28) and can provide an indication whether the overall global fold of a series of a peptide is maintained (29). For a series of structurally related peptides, secondary Hα chemical shifts can be used to identify the location but not the nature of local changes in conformation (29). Secondary Hα chemical shifts were used in the first instance to compare χ-MrIA with its alanine-substituted analogs (Fig. 7). The results indicate that the overall global fold of the χ-MrIA analogs used in this study are conserved compared with native χ-MrIA with the exception of [G6A] MrIA where the overall fold of the peptide appears different. Small local changes are observed for [K8A] MrIA and [H11A] MrIA at the site of the altered residue. For [Y7A] MrIA, a small change in the secondary Hα chemical shift is seen at Lys-8. This is not surprising as Tyr-7 is a relatively large residue that, relative to Ala-7, could influence the chemical environment of Lys-8 and hence differentially influence its Hα chemical shift. In the case of [G6A] MrIA, a comparison of its secondary Hα chemical shifts with χ-MrIA indicates that replacement of this residue causes a significant structural perturbation. Interestingly, introduction of a stereocenter through substitution of Gly-6 with an alanine appears to alter the structural rigidity of [G6A] MrIA. This enhanced structural rigidity for [G6A] MrIA is supported by changes in secondary Hβ shifts for residue Cys-5 where the two Cys-5 β-protons are well separated in [G6A] MrIA. In contrast, the other χ-MrIA analogs investigated in this study all display degenerate β-pro-
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Fig. 7. 3H NMR spectroscopy of χ-MrIA and its alanine-substituted analogs. Secondary Hα chemical shifts (ppm) for MrIA analogs show the similarity in global fold between native χ-MrIA (●) and the [Y7A]- (▲), [K8A]- (▲), [E9A]- (▲), and [H11A]- (▲) analogs and structural perturbation for [G6A]-MrIA (▲). Secondary Hα shifts were derived from TOCSY spectra recorded at 500 MHz and 293 K at a peptide concentration of ~2 mM.

Discussion

The aim of the present study was to investigate the influence that the transporter identity, the co-substrate Na⁺, and individual residues of χ-MrIA have on the ability of the conopeptide to inhibit monoamine transporters. Whether χ-MrIA acted through a site on the NET that was distinct from the classical inhibitors identified by Koppel et al. (2, 35), the non-competitive mode of action distinguishes χ-MrIA from the dopamine and serotonin. The amino acid identity between the NETs of the two species is 93% (14). The NET is expressed transporters to act with twice the potency at the rat over the human isoform. The amino acid sequence homology of Tyr-7, Lys-8, Leu-9, and His-11 of MrIA in structure (7) are shown in Fig. 8.

Although the potency of χ-MrIA for inhibition of uptake and radioligand binding to the expressed rat NET observed here closely matches its reported potency for potentiating noradrenergic contractions in the isolated rat vas deferens (430 nM; 7), we found that the potency of χ-MrIA for inhibition of the binding of [3H]nisoxetine to rat brain was an order of magnitude lower. Given its lower potency in the rat brain, its only partial inhibitory effect and the modest degree of assumed specificity (i.e., nisoxetine- or desipramine-sensitive) binding in the assay, it is perhaps not surprising that McIntosh et al. (9) did not detect any effect of χ-MrIA (10 μM) in their NET binding assay using conditions somewhat similar to those used here. A possible reason for the only partial inhibition of the specific [3H]nisoxetine binding by χ-MrIA is the additional binding of desipramine and nisoxetine to sites in the rat brain other than the NET such as α₁-adrenoceptors (44) or the SERT (30), which are not also targeted by χ-MrIA. Alternatively, the classical NET inhibitors may bind at multiple sites on the NET in a manner reminiscent of the interaction of the cocaine analog RTI-55 and the SERT (45) with χ-MrIA blocking only a subset of these sites. Our finding that χ-MrIA acts as a full inhibitor of the desipramine-sensitive [3H]nisoxetine binding to rat NET-transfected cell membranes (Fig. 2A) discounts this hypothesis or at least reflects a difference in the presentation of the NET in the membranes of native tissues and transfected cells or even the existence of NET subtypes in the rat. The existence of such subtypes could explain the unexpected reduction in the potency of χ-MrIA observed in the brain binding assay.

Norepinephrine transport by the NET has been shown to be dependent on Na⁺, reflecting the co-transport of Na⁺ with the substrate (46). The reduced transport activity caused by lowering of the extracellular Na⁺ concentration is mediated through an increase in the apparent Kᵣ for norepinephrine and a reduction in the V_max. Extracellular Na⁺ not only affects the affinity of the transporter for substrate but also its affinity for inhibitors. It has previously been shown that desipramine and other antidepressants become less effective inhibitors of uptake with reduced extracellular Na⁺ (47), an observation confirmed in this study. χ-MrIA demonstrates the same pattern of Na⁺ dependence. These findings may signify that desipramine and χ-MrIA target the outward-facing (substrate-accessible) configuration of the transporter whose adoption is promoted by extracellular Na⁺ (31). The Na⁺ dependence of the inhibitory action of χ-MrIA stands in contrast to that of cocaine, another natural product that inhibits the NET. Cocaine competes with Na⁺ for binding to the NET, becoming a more potent inhibitor of transport as extracellular Na⁺ decreases (47).
The three-dimensional structure of χ-MrIA has not been determined but appears very similar to that of χ-MrIB (7), a conopeptide whose sequence differs by only a single residue at the N terminus and that displays very similar pharmacology to χ-MrIA. Similar to the majority of conopeptides, χ-MrIA contains multiple cysteine residues that are linked by intramolecular disulfide bonds. These bonds act to bring the cysteine pairs into close proximity in the core of the peptide with residues in the intercysteine regions exposed as loops. Alanine scanning reveals a critical role for residues in the first and largest of χ-MrIA’s two cysteine-bracketed loops in contributing to the activity of the peptide at the NET. Substitution of any of the residues in this region with alanine results in a loss of potency predicted to be in excess of ~600-fold. With the exception of the replacement of Gly-6, the alanine substitutions did not affect the structure of the peptide backbone to any great degree. Tyr-7, Lys-8, Leu-9, and to a lesser extent His-11 therefore seem likely to directly interact with the transporter, whereas Gly-6 probably plays a structural role to allow the correct orientation of the other residues in the loop for better NET binding. The involvement of tyrosine and lysine residues in the high affinity interaction of other peptide toxins with their targets has been reported previously (48, 49), warranting further investigation into χ-MrIA’s use of these residues as high affinity binding determinants in experiments with additional analogs. Phenylalanine was found to be able to largely substitute for Tyr-7, indicating that the hydroxyl group of the tyrosine residue is not of critical importance for binding. This stands in contrast to its role in the interaction of the residues at positions 7–9 and 11 and the N terminus labeled. Positively charged surface is shown in blue, and hydrophobic surface shown in white. The model of χ-MrIA was generated in Insight and was based on the solution structure of χ-MrIB (7) (Protein Data Bank accession number 1IEO) using residue replacement of Val-1 in χ-MrIB to Asn-1 (the corresponding residue in χ-MrIA). The ribbon representation was generated using Insight 2000.1 (50), and the electrostatic surface was generated using GRASP (51) on a Silicon Graphics Octane computer.

FIG. 8. Pharmacophore model of χ-MrIA. A, ribbon representation of χ-MrIA with residues determined to be important for interaction with the NET indicated as follows: Tyr-7 (pink), Lys-8 (blue), Leu-9, and His-11 (red). Disulfide connectivity is shown in orange. B, electrostatic surface of χ-MrIA with residues 7–9 and 11 and the N terminus labeled. Positively charged surface is shown in blue, and hydrophobic surface shown in white. The model of χ-MrIA was generated in Insight and was based on the solution structure of χ-MrIB (7) (Protein Data Bank accession number 1IEO) using residue replacement of Val-1 in χ-MrIB to Asn-1 (the corresponding residue in χ-MrIA). The ribbon representation was generated using Insight 2000.1 (50), and the electrostatic surface was generated using GRASP (51) on a Silicon Graphics Octane computer.

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