Two new classes of conopeptides inhibit the α1-adrenoceptor and noradrenaline transporter

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Cone snails use venom containing a cocktail of peptides ('conopeptides') to capture their prey. Many of these peptides also target mammalian receptors, often with exquisite selectivity. Here we report the discovery of two new classes of conopeptides. One class targets α_1-adrenoceptors (ρ-TIA from the fish-hunting *Conus tulipa*), and the second class targets the neuronal noradrenaline transporter (χ-MrIA and χ-MrIB from the mollusk-hunting *C. marmoreus*). ρ-TIA and χ-MrIA selectively modulate these important membrane-bound proteins. Both peptides act as reversible non-competitive inhibitors and provide alternative avenues for the identification of inhibitor drugs.

Marine snails of the genus *Conus* ('cone snails') are predatory gastropod mollusks found on or near coral reefs in tropical waters throughout the world. They have evolved an elaborate strategy for the rapid immobilization of fish, worms or other mollusks that involves the injection of a complex mixture of bioactive peptides into their prey. These conopeptides are typically 10–30 amino acid residues long and contain multiple intramolecular disulfide bonds. The venom of any single *Conus* species may contain more than 100 different peptides[1]. Conopeptides interfere with neurotransmission by targeting a variety of proteins expressed on the cell surface. Presently, distinct classes of conopeptides are recognized to act at voltage-sensitive Ca^{2+} channels[2], Na^+ channels[3–5], K^+ channels[6,7], nicotinic ACh receptors[8–10], 5-HT_3 receptors[11], NMDA receptors[12], vasopressin receptors[13] and neurotensin receptors[14]. The potential of conopeptides as tools in neuroscience or as therapeutic agents remains largely unexplored, given that the conopeptides characterized to date are estimated to represent only 0.1% of the conopeptides present in the ~500 species of *Conus*[15].

Here we describe the discovery and characterization of two new classes of conopeptides: the ρ-conopeptide class defined by conopeptide TIA from *Conus tulipa*, and the χ-conopeptide class defined by conopeptides MrIA and MrIB from *C. marmoreus* (**Table 1**, **Fig. 1**). Both classes interfere with the action of noradrenaline (NA), a key neurotransmitter in the central and peripheral nervous systems. ^{1}H NMR studies reveal marked differences in the three-dimensional structures of these two-loop peptides. To achieve their different pharmacologies, the ρ-conopeptides use a new combination of amino acids arranged on an α-conopeptide fold (1–3, 2–4 disulfide connectivity), whereas the χ-conopeptides use a different sequence and conopeptide disulfide framework (1–4, 2–3).

Results

Peptide discovery

While searching for α-conopeptides (competitive antagonists of the nicotinic ACh receptor), we isolated and sequenced the polypeptides TIA ($[M + 2H]^{2+}$, observed $m/z = 1195.57$, expected $m/z = 1195.57$; calculated and expected $[M + H]^+$ $m/z = 2390.15$), MrIA ($[M + H]^+$, observed $m/z = 1408.53$, expected $m/z = 1408.53$), and MrIB ($[M + H]^+$ observed $m/z = 1393.52$, expected $m/z = 1393.55$) from the crude venom of *C. tulipa* and *C. marmoreus* collected from the Great Barrier Reef, Australia (**Table 1**, **Fig. 1**). ($[M + H]^+$ and $[M + 2H]^{2+}$ refer to the ionization states of the molecules, and m/z is the mass/charge ratio determined by mass spectrometry.) Surprisingly, these polypeptides had little sequence homology to previously identified conopeptides, despite the cysteine residues being positioned in the linear sequence in a manner reminiscent of α-conopeptides. To allow investigations into their mechanisms of action and three-dimensional structures, chemically identical synthesized forms of these peptides were assembled.

Pharmacology

The likely modes of action of TIA and MrIA were identified from studies of their effects on the biphasic contractile response of the electrically stimulated rat prostatic vas deferens. Both peptides affected the second phase of contraction, which peaks approximately 600 ms after stimulation due to the action of neurally released NA on postjunctional α_1-adrenoceptors[16]. This NA-mediated response was preceded by a faster contraction, which peaked approximately 200 ms after stimulation due to the action of the sympathetic co-transmitter ATP at postjunctional P2X-purinoceptors[17]. TIA and MrIA had opposite effects on the strength of the NA-mediated component of the vas deferens con-

Table 1. Sequence and pharmacological diversity of 2-loop conopeptides.

Class	Name	Sequence	Connectivity	Physiological target
ρ	TIA	FNWR**CC**LIPA**C**RRNHKKF**C***	I–3, 2–4	α_1-adrenoceptor
χ	MrIA	NGV**CC**GYKL**C**HO**C**	I–4, 2–3	noradrenaline transporter
	MrIB	VGV**CC**GYKL**C**HO**C**	I–4, 2–3	
α	GI	E**CC**NPA**C**GRHYS**C***	I–3, 2–4	nicotinic acetylcholine
	PnIB	G**CC**SLPP**C**ALSNPDY**C***	I–3, 2–4	receptors

Cysteines involved in disulfide bonds are labeled I–4 with respect to the linear sequences. *Amidated C-termini. O, 4-hydroxyproline. GI was isolated from *Conus geographus*[8], and PnIB was isolated from *C. pennaceus*[38].

traction. TIA (concentration producing 50% inhibition (IC_{50}), 500 nM) caused a concentration-dependent inhibition of the second phase of the contraction, without affecting the ATP-mediated response (**Fig. 2a**). This inhibition was slowly reversed upon washout with conopeptide-free solution. In the epididymal segment of rat vas deferens, TIA (3 μM and 10 μM) increased the concentration of exogenously applied NA required to produce the half-maximal effect (EC_{50}) by 5.5- and 17.8-fold, and depressed the level of the maximum response to 82% and 42% of the control response, respectively. Because the effects of TIA were not surmountable, the peptide acted as a non-competitive antagonist of NA-induced contractions in the vas deferens. The concomitant decrease in NA potency is explained by the underlying hyperbolic relationship between α_1-adrenoceptor activation and the contractile response in the rat vas deferens[18]. At relatively low concentrations of a non-competitive α_1-adrenoceptor antagonist, part of the receptor pool can be removed without affecting the level of the maximum response. This gives rise to the phenomenon of 'spare receptors.' The EC_{50} for NA is increased in this situation because the fractional receptor occupancy required to generate the half-maximal response is increased with the reduction in the size of the available receptor pool. In addition, the half-maximal response does not occur at 50% receptor occupancy because of the underlying non-linear relationship between receptor activation and response. As the receptor pool is further diminished by increasing concentrations of a non-competitive antagonist, the maximum response to NA is depressed. This reduction in the level of the maximum response is accompanied by a rightward shift of the concentration–response curve (that is, increased EC_{50}). Again, this is due to the increase in the fractional receptor occupancy required by NA to produce the half-maximal response[19].

To define the molecular pharmacology of TIA, we investigated its action on the cloned hamster α_{1B}-adrenoceptor expressed in COS-1 cells. TIA dose-dependently displaced binding of the α_1-adrenoceptor antagonist ^{125}I-HEAT to α_{1B}-adrenoceptors (IC_{50}, 124 nM) (**Fig. 2b**), confirming that TIA is an inhibitor of the α_1-adrenoceptor. Thus, TIA belongs to a previously unknown class of conopeptides we name ρ-conopeptides. This inhibition was non-competitive, as saturation binding studies revealed that ρ-TIA (1 μM) reduced maximum ^{125}I-HEAT binding to α_{1B}-adrenoceptors by 85% without affecting the affinity of the receptor for the radioligand (K_d) (**Fig. 2c**). Thus, TIA acts non-competitively to inhibit the binding of agonists (NA) and antagonists (HEAT) that act directly at the NA binding site[20].

In contrast to ρ-TIA, MrIA, its amidated derivative MrIA-NH_2, and MrIB-NH_2 caused a concentration-dependent increase in the second phase of the contraction of the rat prostatic vas deferens in response to electrical field stimulation (**Fig. 3a**). Again, the ATP-mediated response was unaffected, and the effect of the peptides was reversed upon washout. MrIA-NH_2 (EC_{50}, 430 nM), being 1.5- and 2-fold more potent than MrIA and MrIB-NH_2, respectively, was used to establish the mechanism for this potentiation. Log concentration–response curves to exogenously applied NA were dose-dependently shifted leftward in a parallel manner 3.3- and 7.4-fold by MrIA-NH_2 (1–3 μM), indicating that the sensitivity of the tissue to NA was enhanced by the peptide. However, MrIA-NH_2 (3 μM) had no effect on the responsiveness of the tissue to methoxamine. Because methoxamine is an α_1-adrenoceptor agonist, but not a substrate for the noradrenaline transporter[21] (NET), we hypothesized that MrIA-NH_2 acts to inhibit the NET, the principal route of elimination of NA from the synapse.

To define the molecular pharmacology of MrIA-NH_2, we investigated its action on the cloned rat and human NET expressed in COS-1 cells. The rate of cellular accumulation of ^{3}H-NA via the rat NET was reduced by MrIA-NH_2 (IC_{50} 615 nM) in a dose-dependent manner (**Fig. 3b**). MrIA-NH_2 also inhibited transport of ^{3}H-NA by the human NET (IC_{50}, 1.26 μM). Thus, MrIA-NH_2 is an inhibitor of the NET, making it the first of a previously unknown class of conopeptides we name χ-conopeptides. This action was found to be non-competitive in nature, as χ-MrIA-NH_2 (1 μM) reduced the maximum rate of ^{3}H-NA uptake by the human NET by 42% without affecting the affinity of the transporter for substrate (K_m) (**Fig. 3c**). Of the family of monoamine neurotransmitter transporters, χ-MrIA-NH_2 is selective for the NET, because at 100 μM it did not inhibit the closely related human dopamine and serotonin transporters (data not shown).

A range of tissue assays were used to determine the selectivity of the ρ- and χ-conopeptides. Responses of the guinea pig ileum to nicotine and the mouse phrenic nerve–hemidiaphragm to electrical stimulation were unaffected by ρ-TIA (10 μM) and χ-MrIA-NH_2 (3 μM), indicating that unlike the α-conopeptides,

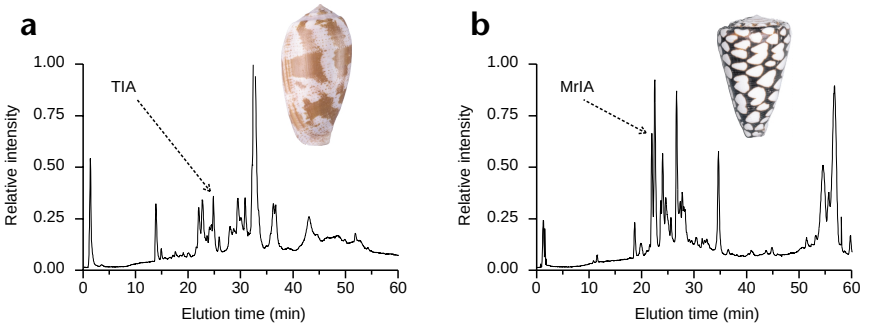

Fig. 1. Liquid chromatography/mass spectroscopy (LC/MS) analysis of crude venom from *C. tulipa* and *C. marmoreus*. (**a**) TIA eluted as a minor component at 24.5 min (Inset *C. tulipa* shell). (**b**) MrIA eluted at 21.8 min, and MrIB eluted as a minor component at 24.2 min (Inset *C. marmoreus* shell). Over 90% of the conopeptides present in the crude venom of these species have not been characterized.

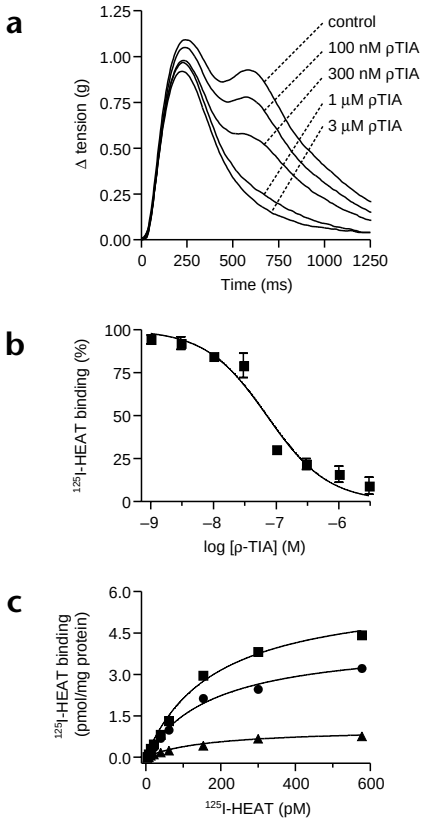

Fig. 2. Mode of action of ρ-TIA. (**a**) Inhibition of electrically evoked contractions of the prostatic portion of the rat vas deferens by ρ-TIA. (**b**) Displacement of ^{125}I-HEAT binding to expressed α_{1B}-adrenoceptor by ρ-TIA. (**c**) Saturation curves for ^{125}I-HEAT binding to the α_{1B}-adrenoceptor expressed in COS-1 cells in the absence (■) and presence of either 30 nM (●) or 1 μM (▲) ρ-TIA. Symbols represent the mean ± s.e.m. of n = 3 separate experiments performed in duplicate. Some error bars are obscured by the symbols.

these peptides do not target neuronal or muscle subtypes of the nicotinic ACh receptor. ρ-TIA and χ-MrIA-NH_2 had no effect on the first component of vas deferens contraction. Thus, both are without effects on the voltage-sensitive ion channels involved in action potential propagation and neurotransmitter exocytosis, which are targeted by certain other conopeptide classes[2,3,7]. Local anesthetic-type activity, such as blockade of neuronal Na^+ channels, has been previously reported for prazosin and other α_1-adrenoceptor antagonists[22,23], and for cocaine[24]. Also unaffected by ρ-TIA (10 μM) were presynaptic α_2-adrenoceptors, of which the activation by NA inhibits evoked contractions in the prazosin-treated rat vas deferens. χ-MrIA-NH_2 did not affect contractions to methoxamine, indicating that the peptide does not act at α_1-adrenoceptors, distinguishing it from some other NET inhibitors, such as the antidepressants desipramine and amitriptyline[25].

Three-dimensional structures

To begin to understand the structural features underlying the biological activity of ρ- and χ-conopeptides, we used ^{1}H NMR techniques to determine the structures of ρ-TIA and a representative χ-conopeptide, χ-MrIB-NH_2. ρ-TIA gave NMR spectra with good NH chemical shift dispersion, indicative of a well-structured molecule. A total of 213 distance restraints derived from 17 intra-residue, 93 sequential, 67 medium and 36 long-range NOEs (nuclear Overhauser effects), 7 dihedral and 19 αH chemical shift restraints were obtained for ρ-TIA from 500 and 750 MHz data. In contrast, χ-MrIB-NH_2 showed peak 'brothering,' indicating the presence of a minor conformation (<10% relative population). However, changing the solvent conditions to 30% acetonitrile/H_2O or 30% TFE/H_2O emphasized the major conformation. Comparable NMR data were also obtained for χ-MrIA and χ-MrIA-NH_2, indicating similar structures among the χ-conopeptides. Like MrIB-NH_2, these peptides showed evidence for minor conformations, most likely associated with *cis–trans* isomerization of the His10–Hyp11 peptide bond. This proposal is supported by a doubling of peaks for residues flanking this bond (His11, Cys13) and those physically close to it (Gly2, Val3) due to nearby disulfide linkages. The major conformer clearly has a *trans* His10–Hyp11 peptide bond based on a strong sequential His10αH→Hyp11δH NOE in all of the χ-conopeptides, but the low intensity and limited number of peaks observed for the minor conformers prevented their definitive characterization. The possibility that configurational isomerization of the disulfide bonds (ProR/ProS forms) also contributes to the minor forms cannot be excluded. However, whatever the cause of the minor forms, they are very similar in overall structure to the major conformer, as the NH chemical shifts of the brothered peaks are all within 0.05 p.p.m. of the major conformer. A total of 82 distance restraints derived from 14 intra-residue, 42 sequential, 3 medium, 23 long range NOEs and 4 dihedral restraints were obtained for the major conformation of χ-MrIB-NH_2 from 500 MHz data.

For both ρ-TIA and χ-MrIB-NH_2, 50 structures were energy-minimized, and the 20 lowest-energy structures were chosen to represent the final structures (**Fig. 4**). ρ-TIA consisted of a stretch of 3_{10} helix between Arg4 to Leu8, a helical turn from Pro9 to Arg12, followed by four nested β-turns between Arg12 and Cys19, which almost comprised another turn of helix. These comprised a type I β-turn between residues 12–15, and three type IV β-turns between residues 13–16, 14–17 and 15–18. ρ-TIA had a backbone pairwise root-mean-square deviation (RMSD) of 0.73 Å (1.94 Å for all heavy atoms), and no NOE violations greater than 0.2 Å (4 violations greater than 0.1 Å).

In contrast to ρ-TIA, χ-MrIB-NH_2 contained no helical elements, and instead formed a small β-hairpin structure. Specifically, χ-MrIB-NH_2 contained a flexible loop between Tyr7 and Leu9, which was presented on the β-hairpin structure, with main-chain H-bonds between Cys4 NH and His11 C=O (all structures), and Gly6 NH and Leu9 C=O (13/20 structures). χ-MrIB-NH_2 had a backbone pairwise RMSD of 1.23 Å (2.90 Å for all heavy atoms) and no NOE violations greater than 0.2 Å (3 violations greater than 0.1 Å). Although the RMSD was higher than is typically seen for larger proteins, it is not unusual for small peptides to exhibit high RMSD values. NOEs are weaker than for larger proteins due to correlation time effects, and small peptides lack a core region that typically gives many NOE contacts. Both factors can lead to lower precision (RMSD), but the structures reported here are sufficient to define the global fold. In addition, χ-MrIB-NH_2 was less defined between residues Tyr7–Leu9, possibly reflecting flexibility in this region. With these residues excluded from the calculation, the backbone RMSD was 0.74 Å.

Discussion

The ρ- and χ-conopeptides are the first examples of peptides that act selectively at α_1-adrenoceptors and the NET. Both classes act allosterically through sites distinct from those targeted by nora-

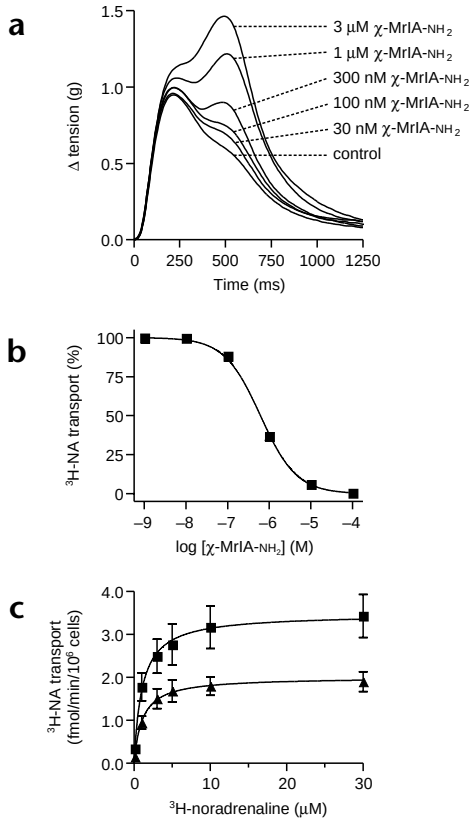

Fig. 3. Mode of action of χ-MrIA-NH_2. (**a**) Enhancement of electrically evoked contractions of the prostatic portion of the rat vas deferens by χ-MrIA-NH_2. (**b**) Inhibition of ^{3}H-NA uptake into COS-1 cells via expressed rNET by χ-MrIA-NH_2. (**c**) Kinetics of ^{3}H-NA transport by hNET expressed in COS-1 cells in the absence (■) and presence of 1 μM χ-MrIA-NH_2 (▲). Symbols represent the mean ± s.e.m. of n = 3 separate experiments performed in duplicate. Some error bars are obscured by the symbols.

drenaline. They are structurally distinct from the various other classes of small molecule inhibitors of the α_1-adrenoceptor and NET that act competitively at the site of NA interaction, many of which are important therapeutic agents. Both ρ-TIA and χ-MrIA-NH_2 lack the common and often therapeutically limiting pharmacology of α_1-adrenoceptor antagonists (α_2-adrenoceptor and Na^+-channel inhibition) and NET inhibitors (α_1-adrenoceptor and muscarinic ACh receptor antagonism), and thus may be useful clinically. Given the physical characteristics of conopeptides, we expect that these peptides will not cross the cell membrane, and that their site of action is on the extracellular surface of these proteins. Previously, a peptide with sequence identical to χ-MrIA was found to have antinociceptive activity when administered intrathecally to mice[26]. This is not unexpected given the role of NA in antinociception[27], and indicates that χ-MrIA may have potential as an analgesic.

ρ-TIA, with a 4/7 two-loop framework, adopts the canonical α-conopeptide fold with a 1–3, 2–4 disulfide bond connectivity, as exemplified by PnIB (**Fig. 4**). The ρ-TIA structure is also related to the published X-ray structures for α-conopeptides [Tyr^{15}]-PnIA, [Tyr^{15}]-PnIB and [Tyr^{15}]-EpI, with a backbone RMSD of approximately 1.0 Å over the two loops[28–30]. Thus ρ-conopeptides achieve their pharmacology by displaying different side-chain elements on a peptide backbone that can also be used to target nicotinic ACh receptors. In contrast, χ-MrIB-NH_2 has a distinctly different structure. The presence of a Cys5 αH to Cys13 NH NOE, strong Cys4 βH to Cys13 αH and βH NOEs, and Cys5 αH to Cys10 αH NOE is not compatible with the canonical 1–3, 2–4 disulfide bond connectivity, but is instead indicative of a 1–4, 2–3 disulfide bond connectivity. This connectivity was also shown chemically by selective reduction/alkylation studies (data not shown), and by the selective synthesis of the three possible structural isomers[26,31]. Thus, χ-conopeptides achieve their pharmacology through a conopeptide structure that uses different side-chain elements and a previously unidentified conopeptide backbone. Although we assume that the observed activity for the χ-conopeptides arises from the major (>90%) conformation described here, minor forms, most likely associated with *cis–trans* isomerisation of Hyp11, do occur in solution, and could potentially contribute to the biological activity. In any case, these minor forms are very similar to the major conformation and exhibit the same connectivity of disulfide bonds. Given that this is a previously unknown disulfide arrangement for native conopeptides, it is not surprising that χ-MrIB-NH_2 superimposed poorly with α-conopeptides such as GI (**Fig. 4**). For example, the heavy atom RMSD for the cysteine residues of χ-MrIB-NH_2 and GI was only 4.02 Å. These high-resolution three-dimensional structures of ρ- and χ-conopeptides provide templates to guide the design of peptidomimetic inhibitors of α_1-adrenoceptors and the NET.

These two conopeptide classes open up alternative avenues for modulating α_1-adrenoceptors and the NET. Because these peptides are non-competitive inhibitors, they seem to act allosterically at modulatory sites distinct from the site of NA interaction, presumably at the extracellular loops of these membrane-bound proteins. These modulatory sites on the α_1-adrenoceptor and the NET may also be sites where endogenous mammalian peptide inhibitors act. An example of such an endogenous modulatory peptide is 5-HT-moduline, a non-competitive inhibitor of the

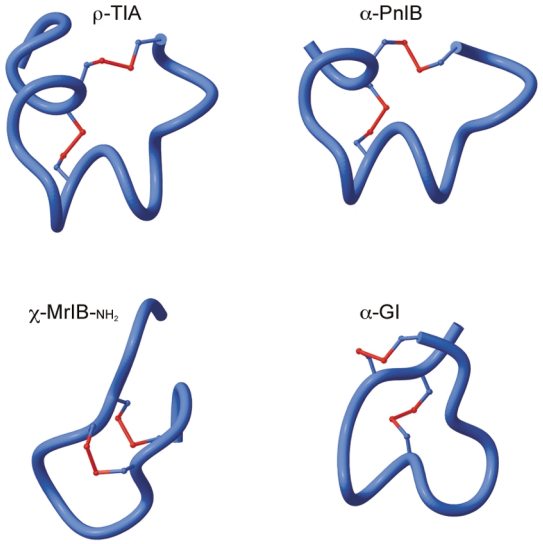

Fig. 4. NMR-derived structures of ρ-TIA and χ-MrIB-NH_2. The NMR structure of α-conopeptides PnIB and GI are shown for comparison. Based on loop size, ρ-TIA (4/7) and χ-MrIB-NH_2 (4/2) are closest in structure to α-conopeptides PnIB (4/7) and GI (3/5), respectively. Disulfide bonds are shown in red.

5-$HT_{1B/D}$ G-protein-coupled receptor[32]. It remains to be seen if non-competitive modulatory sites are a common feature across G-protein-coupled receptors and transporters.

The involvement of ρ-TIA and χ-MrIA in prey capture is less clear, as neither conopeptide alone was toxic to *Gambusia affinis* at 25 nmol/g. The peptides have not been tested for biological activity in mollusks. It is possible that these peptides act (perhaps even at a different physiological target in the prey species) in a 'cabal' with other bioactive peptides in the venom to produce a synergistic effect that aids prey capture[33]. The two-loop CCX_nCX_nC peptides, in which X defines residues comprising the intercysteine loops, seem to represent a 'privileged' structural class that can access diverse pharmacology, reminiscent of the four-loop $CX_nCCX_nCX_nCX_nC$ peptides, which target voltage-sensitive Ca^{2+} (ω-conopeptides)[2], Na^+ (μO-conopeptides)[5] and K^+ (κ-conopeptides)[7] ion channels. However, the two-loop structural class act at membrane proteins targeted by the neurotransmitters NA (ρ- and χ-conopeptides) and ACh (α-conopeptides), rather than at voltage-sensitive ion channels.

Methods

Peptide isolation. The peptides were purified to homogeneity, and their primary structures were determined by Edman sequencing, following procedures previously described[34]. ρ-TIA, χ-MrIA and χ-MrIB were identified in crude venom from *C. tulipa* and *C. marmoreus* (0.6 mg/mL, 15 and 5 μL injected, respectively) by reverse-phase HPLC using a C_{18} column (3.5 μm, 2.1 × 50 mm Zorbax 300 SB, Palo Alto, California) eluted with a 1% linear gradient from 0 to 60% B (A = 0.1% formic acid, B = 90% acetonitrile/0.1% formic acid) using a HP1100 pump. The eluant was continuously monitored with a QSTAR pulsar (PE-Sciex, Foster City, California) mass spectrometer. Mass spectrometry and sequence data indicated that the C-terminus of ρ-TIA is amidated, whereas χ-MrIA and χ-MrIB are the free-acid forms.

Peptide synthesis. ρ-TIA (amidated), χ-MrIA (amidated and free-acid) and χ-MrIB (amidated) were synthesized using Fmoc and Bmoc chemistry, and oxidized in $NaHCO_3$, pH 8.0[34]. The final overall yield was 65% for TIA and 30% for MrIA, with purity greater than 98% by HPLC. On coinjection onto a Waters C_{18} symmetry column (Milford, Massachussets), TIA (amidated) coeluted with native TIA, and HPLC purified MrIA (free acid), but not the amidated form of MrIA, coeluted with native MrIA.

Rat vas deferens. Segments of *vasa deferentia* from male Wistar rats (250–350 g) were mounted under a tension of 0.5 g in 5 mL organ baths containing 119 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 5.5 mM glucose, 2.5 mM $CaCl_2$ and 0.026 mM EDTA (pH 7.4, 37°C, bubbled with 5% CO_2:95% O_2). Bisected prostatic segments were field-stimulated with single electrical pulses of 55-V amplitude and 1-ms duration generated by a Grass S44 stimulator (Quincy, Massachussets) at 3-min intervals. Isometric contractions were recorded digitally on a Power Macintosh computer with a MacLab/8s data acquisition system (ADInstruments, Sydney, Australia). The resulting contractions could be abolished by tetrodotoxin (0.1 μM), indicating a neural origin. Drugs were added cumulatively to the organ. All animal experiments were conducted with the approval of the University of Queensland Animal Experimentation Ethics Committee.

To examine if ρ-TIA affected presynaptic α_2-adrenoceptors, electrical pulses as described above were delivered at 20-s intervals to the prostatic portion of vas deferens treated with prazosin (0.5 μM). Cumulative concentration–response curves for the inhibitory effect of NA were established before and 20 min after addition of ρ-TIA (10 μM). To determine whether ρ-TIA affected postjunctional contractile responses to NA, we used unstimulated bisected epididymal segments. Cumulative concentration–response curves to NA were established in the absence or presence of ρ-TIA (3 μM or 10 μM applied 20 min before NA). The bisected epididymal segments were also used to establish concentration–response curves to NA and methoxamine in the absence and presence of χ-MrIA-NH_2. χ-MrIA-NH_2 (1 μM or 3 μM) was applied 20 min before cumulative additions of NA or methoxamine, which contract the smooth muscle via postjunctional α_1-adrenoceptors.

Mouse phrenic nerve–hemidiaphragm. Left and right hemidiaphragm were dissected from male Quackenbush mice (20–30 g), mounted under a tension of 1.0 g in 5 mL organ baths containing 135.0 mM NaCl, 5.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 11.0 mM glucose, 15.0 mM $NaHCO_3$ and 1.0 mM KH_2PO_4 (pH 7.4, 37°C, bubbled with 5% CO_2:95% O_2). Field stimulation of the phrenic nerve with 3-V pulses of 0.2-ms duration delivered at 20-s intervals evoked contractions recorded as described above. We examined the effect of ρ-TIA (10 μM) and χ-MrIA-NH_2 (3 μM) on the contractions.

Guinea pig ileum. Segments of ilea (~1.5 cm) from male guinea pigs (280–430 g) were mounted under a resting tension of 1.0 g in 5 mL organ baths containing 136.9 mM NaCl, 2.68 mM KCl, 1.84 mM $CaCl_2$, 1.03 mM $MgCl_2$, 5.55 mM glucose, 11.9 mM $NaHCO_3$, 0.45 mM KH_2PO_4 and 10 μm indomethacin (pH 7.4, 37°C, bubbled with 5% CO_2:95% O_2). Non-cumulative doses of nicotine (4 μM) were added until reproducible contractile responses were obtained. Tissue was then exposed to the ρ-TIA (10 μM) or χ-MrIA-NH_2 (3 μM) for 25 min and nicotine was again applied.

^{125}I-HEAT binding. Membranes from COS-1 cells (ATCC, Manassas, Virginia) grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and transiently transfected with plasmid DNA encoding the hamster α_{1B}-adrenoceptor were prepared as described previously[20]. For radioligand binding studies, we used duplicate tubes containing 80 pM ^{125}I-HEAT, COS-1 membranes and various concentrations of ρ-TIA, in HEM buffer (20 mM HEPES, 1.5 mM EGTA, 12.5 mM $MgCl_2$, pH 7.5). For saturation binding studies, ^{125}I-HEAT was added to 575 nM in the presence and absence of TIA. Total reaction volume was 250 μL. Nonspecific binding (5–10% of total) was determined by the inclusion of phentolamine (10^{-4} M). After 80 min of incubation at room temperature, the reactions were stopped by the addition of ice-cold HEM buffer and were transferred onto Whatman GF/C glass filters (Kent, UK).

Uptake of ^{3}H-NA. COS-1 cells grown in 24-well plates containing DMEM and 10% fetal bovine serum were transiently transfected with plasmid DNA encoding the rat[35] or human NET[36] using Lipofectamine 2000 reagent (Gibco, Carlsbad, California). Assays were done at room temperature, 24 h after transfection in transport buffer containing 125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 25 mM HEPES, 5.55 mM D-(+)-glucose, 1.02 mM ascorbic acid, 10 μM U-0521 and 100 μM pargyline, pH 7.4. The cells were exposed to various concentrations of χ-MrIA-NH_2 or desipramine (10^{-5} M; for the determination of nonspecific accumulation) for 15 min before ^{3}H-NA (100 nM, supplemented with unlabeled NA as required) was added for either 8 (rat NET) or 20 min (human NET). ^{3}H-NA was added at concentrations up to 30 μM to determine the kinetics of NA transport by hNET expressed in COS-1 cells. The solution was then rapidly removed, and the cells were washed with ice-cold PBS. The cells were lysed with 0.1% Triton-X-100 in 10 mM Tris·HCl (pH 7.5) for 90 min, and the cell lysate was taken for scintillation counting.

Drugs. The following drugs were used: desipramine hydrochloride, indomethacin, methoxamine hydrochloride, nicotine hydrogen tartrate (–)-noradrenaline bitartrate (NA), pargyline, tetrodotoxin (Sigma, St. Louis, Missouri); phentolamine mesylate (Research Biochemicals International, Natick, Massachussets); U-0521 (Biomol, Plymouth Meeting, Pennsylvania); ^{125}I-HEAT, ^{3}H-NA (NEN Life Science, Boston, Massachussets).

Statistics and data analysis. Data are expressed as means ± s.e.m. of results obtained from 3–6 separate experiments. Student's two-tailed *t*-test or analysis of variance was used for statistical evaluation; values of *p* less than 0.05 were considered significant. Sigmoidal concentration–response curves for the calculation of EC_{50} or IC_{50} values were fit by non-linear regression using Prism software (GraphPad, San Diego, California).

NMR studies. NMR experiments were typically done on 2–5 mM peptide in either D_2O or 90% H_2O/10%D_2O at pH 3.5 and 278–285 K. Additional data on χ-MrIA-NH_2 were obtained in 30% d_3-acetonitrile/10% D_2O/60% H_2O and 30% TFE/10% D_2O/60% H_2O. Spectra were acquired on ARX 500 MHz or AVANCE 750 MHz spectrometer (Bruker, Billerica, Massachussets) equipped with a shielded gradient unit, and referenced to a DSS internal standard. Spectra were processed using UXNMR or XWIN-NMR (Bruker), and analyzed in Felix (Hare Research, Bothell, Massachussets). NOE crosspeaks were assigned as strong (1.8–2.7 Å), medium (1.8–3.5 Å) or weak (1.8–5.0 Å). NOE peaks observed at long mixing times (≥400 ms) but not at shorter mixing times were classed as very weak (1.8–6 Å). Pseudo-atom corrections of 1.5 Å for methyl, 1.0 Å for methylene, and 2.0 Å for phenyl and tyrosine ring protons were added. Structures were calculated from distance and angle restraints using the torsion-angle dynamics/simulated annealing protocol as previously described[37]. After the initial structures showed discrepancies due to the unusual disulfide connectivity in χ-MrIB-NH_2, the simulated annealing protocol was modified to first calculate the structures without disulfide bonds. With this protocol, 41/50 structures had the preferred disulfide connections 4–13 and 5–10. Incorporating this disulfide connectivity into the simulated annealing protocol improved structural convergence.

The coordinates for ρ-TIA and χ-MrIB-NH_2 have been deposited with the Research Collabatory for Structural Biology (RCSB) with accession codes RCSB013213 (PDB ID 1IEN) and RCSB013214 (PDB 1IEO).

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