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MrIC, a Novel α-Conotoxin Agonist in the Presence of PNU at Endogenous α7 Nicotinic Acetylcholine Receptors

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Supporting Information

ABSTRACT: α-Conotoxins are competitive antagonists of nicotinic acetylcholine receptors (nAChRs). Their high selectivity and affinity for the various subtypes of nAChRs have led to significant advances in our understanding of the structure and function of these key ion channels. Here we report the discovery of a novel 4/7 α-conotoxin, MrIC from the venom duct of *Conus marmoreus*, which acts as an agonist at the endogenous human α7 nAChR in SH-SY5Y cells pretreated with PNU120596 (PNU). This unique agonist activity of MrIC at α7 nAChRs may guide the development of novel α7 nAChR modulators.

The marine cone snails of the genus *Conus* comprise a family of approximately 700 species that produce venom made of a complex toxin repertoire to capture prey and defend against predators.[1,2] The best characterized components in their venom are the disulfide rich peptides termed conotoxins.[3] The conotoxins have attracted the attention of researchers because of their high affinity and selectivity for a range of ion channels, receptors, and transporters in the neuromuscular system. As a result of their unique globular structures, *Conus* venom peptides are invaluable pharmacological tools with potential as drug leads.[4] Of the 10 conotoxins assessed preclinically, four have entered clinical trials,[5] with ω-conotoxin MVIIA (Prialt) from *Conus magus* marketed for the treatment of severe neuropathic pain.[6]

Venom peptides of the α-conotoxin family are small (12−19 amino acid residues) and highly stable because of the presence of two disulfide bonds (i.e., Cys1−Cys3 and Cys2−Cys4).[7] The sizes of the loops between the cysteine residues further divide the family into five major subfamilies, α3/5, α4/3, α4/4, α4/6, and α4/7.[8,9] The vast majority of α-conotoxins characterized are competitive inhibitors of nicotinic acetylcholine receptors (nAChRs),[10−13] with α3/5 conotoxins selectively targeting the muscle nAChRs and α4/3, α4/4, α4/6, and α4/7 conotoxins being specific antagonists for neuronal nAChRs found in the central nervous system.[14]

In this study, we report the synthesis and pharmacological characterization of a novel α-conotoxin, MrIC. Our results reveal that MrIC acts as a co-agonist with PNU at the endogenous α7 nAChR and shows no detectable effect at α3β2 and α3β4 nAChRs.

The sequence of this novel 4/7 α-conotoxin was identified in the venom gland transcriptome of *Conus marmoreus*.[15] The signal peptide of the precursor (Mr1.7) was 100% homologous to the signal peptide of the A superfamily, and its sequence was expressed at a moderate mRNA level (20 cDNA reads) (Figure 1 of the Supporting Information). At the peptide level, several variants were identified in the venom (Table 1). The major mature peptide (Mr1.7a) was amidated and had only one residue before the first cysteine residue. The second most abundant peptide (Mr1.7b) was in the free acid form with an extra C-terminal serine residue, two hydroxyproline residues, and a three-residue N-terminal tail. The third variant (Mr1.7c or MrIC) was a minor component in the venom and identical to Mr1.7a except for an extra proline residue at the N-terminus. The sequence of MrIC is compared with those of other homologous α-conotoxins, with conserved residues highlighted in gray (Table 2).

Table 1. Variants of Mr1.7 Identified at the Protein Level

Peptide	Sequence
Mr1.7a	ECCTHPACHVSNPELC-NH_2
Mr1.7b	ROECCTHOACHVSNPELCS-OH
Mr1.7c (MrIC)	PECCTHPACHVSNPELC-NH_2

O indicates hydroxyproline

All three peptide variants were assembled using Fmoc chemistry[16] and air oxidized under mild basic conditions after cleavage to produce two dominant isomers in a ratio of ∼1:1.

Table 2. Sequence Homology of Related α-Conotoxins

Peptide	Sequence
MrIC	PECCTHPACHV SNPELC-NH_2
PeIA	GCCSHPACS VNHPELC-OH
RegIIA	GCCSHPACNVNNPHIC-NH_2
OmIA	GCCSHPACNVNNPHIC-NH_2
GID	IRDECCSNPACRVNNPHVC-OH

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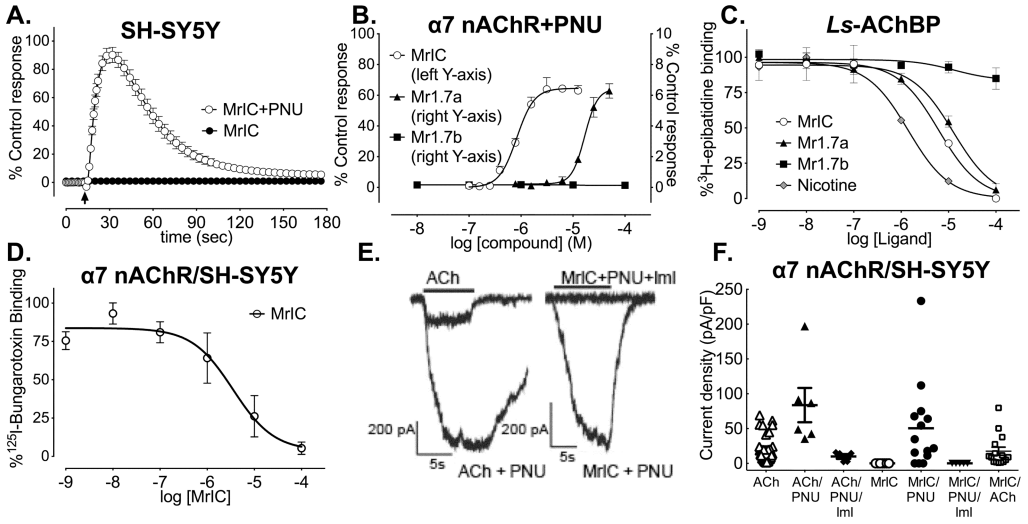

Figure 1. Action of MrIC and analogues on human SH-SY5Y neuroblastoma cells and acetylcholine binding protein *Ls*-AChBP. (A) MrIC (30 μM) elicits a Ca^{2+} response in SH-SY5Y cells preincubated for 30 min with the α7 nAChR-specific modulator PNU at 10 μM (average response of 71.3 ± 3.3% relative to that of 30 μM choline). (B) Concentration−response curves of MrIC analogues mediated increases in the intracellular Ca^{2+} concentration in SH-SY5Y cells in the presence of PNU in comparison with that with choline. (C) Displacement of binding of [^{3}H]epibatidine to *Ls*-AChBP. (D) Displacement of binding of [^{125}I]bungarotoxin to α7 nAChR in SH-SY5Y membranes. (E) Whole-cell patch-clamp electrophysiology of SH-SY5Y cells. (F) Summary of electrophysiological actions in SH-SY5Y cells.

The two isomers eluted ~3% B apart on a C_{18} column, the late-eluting isomer of Mr1.7a co-eluting with native Mr1.7a (Figure 2A−D of the Supporting Information). Co-elution was also observed for native Mr1.7b and Mr1.7c with the late-eluting isomers of synthetic Mr1.7b and MrIC. Circular dichroism spectra in aqueous solution revealed that only the isomers that co-eluted with the native peptide maintained an α-helical content similar to that of a typical 4/7 α-conotoxin, while the earlier-eluting fold lost this helical propensity. Addition of TFE failed to further enhance helicity, indicating that the native fold readily adopted a stable helical structure (Figure 2E,F of the Supporting Information).

The functional activity of Mr1.7a, Mr1.7b, and MrIC was assessed using a high-throughput Ca^{2+} FLIPR assay at α7 nAChR and $\alpha3\beta2/\alpha3\beta4$ nAChR endogenously expressed in human SH-SY5Y neuroblastoma cells.[17] None of the peptides significantly inhibited choline-induced α7 nAChR or nicotine-induced $\alpha3\beta2/4$ nAChR responses at concentrations of up to 100 μM (data not shown), indicating no antagonist activity at these receptor subtypes. However, MrIC acted as an almost full α7 nAChR agonist (pEC_{50} of 5.78 ± 0.13; average response of 71.3 ± 3.3% relative to that of 30 μM choline) in the presence of the α7-selective allosteric modulator PNU (Figure 1A). This response was inhibited by the competitive nAChR antagonist methyllycaconitine (10 μM), and neither MrIC or PNU alone evoked a detectable Ca^{2+} response (n = 10; data not shown). In contrast, Mr1.7a acted as a weak partial agonist at α7 nAChR (pEC_{50} of 4.8 ± 0.03; maximal response of 6.4 ± 0.4% compared to that of 30 μM choline), whereas Mr1.7b was inactive. No $\alpha3\beta2/4$-mediated responses were elicited by Mr1.7a, Mr1.7b, or MrIC, indicating this agonist effect was specific to PNU-modified α7 nAChRs in SH-SY5Y cells (Figure 1A,B).

Consistent with a direct effect on nAChRs, MrIC and Mr1.7a displaced binding of [^{3}H]epibatidine to *Ls*-AChBP with pIC_{50} values of 5.2 ± 0.3 and 4.9 ± 0.1, respectively, while Mr1.7b had no detectable activity at concentrations of up to 100 μM (Figure 1C). MrIC also displaced binding of [^{125}I]bungarotoxin to α7 nAChR in SH-SY5Y membranes with a pIC_{50} of 5.4 ± 0.3, matching its agonist potency in this cell line (Figure 1D).

To confirm this unique effect of MrIC, electrophysiological studies were performed on SH-SY5Y cells. Acetylcholine (ACh, 30 μM) elicited a small inward current (22.3 ± 4.7 pA/pF) that was attenuated to 9.8 ± 3.0 pA/pF (n = 5) in the presence of the nAChR antagonist α-conotoxin ImI (ImI), indicating contributions from α7,[18] and α3-containing nAChRs. Consistent with expression of homomeric α7 nAChR, the α7-specific allosteric modulator PNU (10 μM) enhanced the size of the ACh-induced current (Figure 1E,F). Consistent with our findings from Ca^{2+} signaling studies, MrIC (30 μM) in the presence of PNU elicited large inward currents that were completely abolished by the α7-specific α-conotoxin ImI (Figure 1E), confirming the effect of MrIC was α7-specific. PNU or MrIC alone (n = 6) again failed to produce a detectable inward current (Figure 1F), and MrIC (30 μM) had no significant effect on currents induced by 30 μM ACh [83 ± 8% of ACh alone; n = 15; p = 0.5 (Figure 1F)]. In contrast, MrIC acted as a simple antagonist at human α7 nAChRs heterologously expressed in *Xenopus* oocytes (pIC_{50} of 5.84 ± 0.03; n = 3), indicating significant functional differences of unknown origin exist between neuronal and oocyte-expressed α7 nAChRs (Figure 3 of the Supporting Information).

Prior to this work, most α-conotoxins were described as antagonists of the nicotinic acetylcholine receptors, though Possani et al. reported two α-conotoxins, SrIA and SrIB, which at low concentrations produced weak partial agonist effects on nAChRs.[19] Here we observed that MrIC is an almost full agonist at the endogenous human α7 nAChR in the presence of PNU, with no activity at endognous $\alpha3\beta2$ and $\alpha3\beta4$ nAChRs in SH-SY5Y cells. α-Conotoxins are characterized by a well-defined consensus structure comprising a helical region centered around the third cysteine residue.[9] A conserved hydrophobic patch in the first loop determines binding, and a more variable second loop contributes to selectivity through pairwise interactions with different nAChR subunits.[12] A comparison of the sequence of MrIC with those of other α-

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conotoxins shows that MrIC has an unusual extended N-terminus comprising a proline and glutamic acid. A similar extended N-terminus is found in α-conotoxin GID, yet GID is a competitive nAChR antagonist and has no agonist effect with PNU (data not shown). Interestingly, the interaction of MrIC with α7 nAChR was reduced with a truncated (Mr1.7a) or elongated (Mr1.7b) N-terminus. Because both Mr1.7a and Mr1.7b have an exposed and charged N-terminal residue [as opposed to the hydrophobic Pro residue in MrIC (Figure 4 of the Supporting Information)], it appears that specific hydrophobic interactions between the conotoxin N-terminus and the receptor favor co-agonist activity. The co-agonist activity of MrIC is unusual because a selection of other α-conotoxins (RgIA, MI, ImI, Mr1.1, Ca1.1, TxIA, PnIA, AuIB, and EpI at 10 μM) had no co-agonist activity in the presence of PNU in SH-SY5Y cells (FLIPR data not shown). Understanding the structure−activity relationship and mode of nAChR activation by MrIC may help guide the development of novel α7 nAChR modulators with potential for the treatment of a range of neurological disorders.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, SH-SY5Y assay, Mr1.7 cDNA sequence, oocyte experiments, synthesis, structural figures, and further FLIPR discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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