A new opioid peptide predicted from cloned cDNAs from skin of *Pachymedusa dacnicolor* and *Agalychnis annae*

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Abstract We have isolated a cDNA encoding a precursor of dermorphin from the skin of *Pachymedusa dacnicolor*. Besides four copies of this opioid peptide, the deduced sequence also contains the genetic information for a novel peptide Tyr-Ile-Phe-His-Leu-Met-Asp-NH_2. This differs from Met-deltorphin by the presence of Ile at position 2. In a related precursor from the skin of *Agalychnis annae*, the sequence of this peptide is in the 3′-untranslated region of the cloned cDNA. From earlier results we predict that in skin peptides the second residue is D-allo-Ile. We have synthesized this and related peptides with different D-amino acids, and determined their δ agonist activity. The peptide with D-nor-Leu binds with high affinity to δ receptors, while that with D-allo-Ile is about 100 times less active.

Key words: Amphibian skin; Opioid peptide; Deltorphin; *Phyllomedusa*

1. Introduction

Numerous peptides have been isolated from the skin of frogs belonging to the subfamily Phyllomedusinae [1,2]. Of particular interest are a group of opioid peptides, dermorphins and deltorphins, which all contain a D-amino acid at the second position [3]. By a combination of peptide isolation, pharmacological tests and cDNA cloning, more than half a dozen such peptides have been characterized. The dermorphins and deltorphins mostly have high affinity and selectivity for μ or δ opioid receptors, respectively.

The subfamily of Phyllomedusinae has been divided further into three groups, the *Phyllomedusa*, the *Agalychnis* and one *Pachymedusa* species [4]. In previous studies, only a few of the more than 30 *Phyllomedusa* species have been investigated. In addition, dermorphin has been isolated from skin extracts of *Agalychnis callydrias* [5]. In an attempt to search for new opioid peptides, we have studied two additional species, *Pachymedusa dacnicolor* and *Agalychnis annae*. We have searched cDNA libraries prepared from skin of these two species using sequence information from cDNAs encoding dermorphin and deltorphin precursors from *Ph. sauvagei* and *Ph. bicolor* [6,7]. Here we describe a new deltorphin predicted from cDNA sequences. We also present the biological activity of this new peptide and some of its relatives.

2. Materials and methods

2.1. Frogs, materials

A. annae from Costa Rica and *P. dacnicolor* from Southern Mexico were bred in captivity by Christian Proy (Vienna). Synthetic peptides were obtained from Dr. R. Frank (University of Heidelberg).

2.2. RNA preparation and cDNA synthesis

Frog skin was immediately frozen on dry ice and stored at −70°C. Total RNA was isolated using guanidinium thiocyanate [8] as described [6]. First strand cDNA was synthesized with 5 μg total RNA using the primer adaptor GGAATTCTCGAGCTCAAGC(T_{18}) and reverse transcriptase (Superscript RNase H−, Gibco-BRL) as recommended by the supplier. The second strand was synthesized with polymerase I and RNase H (Boehringer Mannheim) [9]. After blunt ending, double stranded adaptors were ligated to the cDNA (Marathon cDNA Amplification Kit, Clontech Laboratories). The reaction mixture was diluted 1:50 with water and stored at −20°C.

2.3. Amplification of cDNA ends and sequence analysis

PCR amplification was performed using the degenerate sense and antisense primers derived from the sequence encoding dermorphin (YAFGYPS) [6]. For the 3′-RACE protocol, the downstream gene specific primer and Ada-4 (hybridizing to the cDNA synthesis primer Ada-3) were used. The 5′-RACE was performed with the upstream core primer and AP-1 (hybridizing to the marathon adaptor). Cycling parameters for amplification of adaptor ligated cDNAs were according to Chenchik et al. [10].

Amplified fragments were subcloned into the pBluescript II vector (Stratagene). Sequencing reactions were performed with the thermosequenase kit (Amersham) and IRD-41 fluorescent labeled primers (MWG Biotech) and afterwards analyzed on a LI-COR automated DNA sequencer 4000 (MWG Biotech). Only cDNAs that were identical in at least two separate PCR experiments were used for further characterization. DNA sequences were deposited in the EMBL data base (accession numbers for AJ005443: *Pachymedusa dacnicolor* mRNA for opioid peptide; and AJ005444: *Agalychnis annae* mRNA for opioid peptide, partial.)

2.4. Pharmacological tests

The biological activity of different peptides was tested on isolated preparations of electrically stimulated mouse vas deference (MVD) and guinea pig ileum (GPI) that are rich in δ and μ opioid receptors, respectively [11]. Agonists were evaluated for their ability to inhibit the electrically evoked twitch. The results were expressed as IC_{50} values obtained from concentration-response curves [12]. Values are expressed as mean ± S.E.M. from at least five tissues samples.

Binding of peptides to δ, μ, and κ opioid receptors was assayed on crude membranes from rat brain, guinea pig brain, and cell lines as described previously [11].The δ binding sites were labeled with [^{3}H][D-Ala^2,Asp^4]deltorphin (deltorphin I, 0.3 nM) on rat brain membranes and with [^{3}H][D-Ala^2,Glu^4]deltorphin (deltorphin II, 0.3 nM) on NG-108/15 cells (a murine neuroblastoma×glioma hybrid cell line which expresses high levels of δ receptors) and CHO cells which had been transfected with the cloned mouse δ opioid receptor [13]. The μ sites were labeled with [^{3}H][D-Ala^2,MePhe^4,Glyol^5]enkephalin ([^{3}H]DAGO, 0.5 nM) in rat brain. The κ sites were labeled with [^{3}H]U-69,593 (1 nM) in guinea pig brain. Competition curves were determined in triplicate. The inhibition constant (K_i) of the various peptides was calculated from the curves with the computer program Ligand [14]. Data obtained from three independent measurements are presented as the arithmetic mean ± S.E.M.

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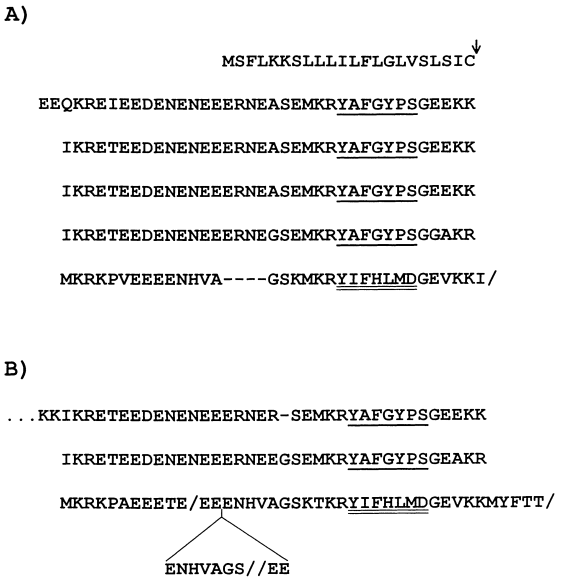

Fig. 1. Amino acid sequences deduced from cloned cDNAs from skin of *Pachymedusa dacnicolor* (A) and *Agalychnis annae* (B). Dermorphin sequences are underlined, the new peptide is double underlined. The end of the signal peptide is marked by an arrow, gaps to maximize internal similarities by dashes (-), stop codons by slashes (/). In the *Agalychnis* sequence, an insertion with two in-frame stop codons is present.

3. Results

The precursors for dermorphins and deltorphins all contain several copies of the mature peptides [6,7]. This was also found to be the case for *P. dacnicolor* and *A. annae*. The amino acid sequence of a precursor as deduced from a cDNA cloned from skin of *P. dacnicolor* is shown in Fig. 1A. This precursor starts with a putative signal peptide and then contains four copies of dermorphin separated by almost identical spacer peptides. Close to the carboxyl end, the sequence of a novel peptide is present. Flanked by the same type of sequences as the dermorphin copies, this peptide has the structure Tyr-Ile-Phe-His-Leu-Met-Asp. Except for the Ile in position 2, this peptide is identical to Met-deltorphin, which was also originally predicted from the sequence of a cloned cDNA [7] and subsequently isolated from skin extracts [15–17].

A cDNA encoding a fragment of a related precursor could be isolated from skin of *A. annae* (Fig. 1B). In the common region the nucleotide and the deduced polypeptide sequence are about 80% identical. However, three in-frame codons are present after the last dermorphin copy. Consequently, the conserved sequence of the peptide related to Met-deltorphin is now located in the 3′-untranslated region of the mRNA encoding the *A. annae* precursor.

Opioid peptides from *Phyllomedusa* skin all contain a D-amino acid at the second position, which is derived from the corresponding L-form in the precursor. In the case of L-isoleucine, a change of chirality of the α carbon yields D-allo-isoleucine (D-aIle). This amino acid has indeed been detected in peptides from skin of *Bombina variegata* [18]. It can thus be assumed that the peptide predicted from the cloned cDNAs has the amino-terminal sequence Tyr-D-aIle-Phe.

We have tested several synthetic homologous of Met-deltorphin which contained either D-allo-isoleucine, D-leucine, D-norleucine (D-nLeu) or D-valine as the second amino acid. The potency, the receptor affinity and selectivity of these peptides have been measured by bioassays on isolated organ preparations and by binding to crude membrane preparations (Table 1). In MVD preparations, the peptide with D-nLeu ($IC_{50} = 0.76 \pm 0.1$ nM) was about three times more active than Met-deltorphin ($IC_{50} = 2.5 \pm 0.3$ nM); conversely, the IC_{50} for the putative natural peptide with D-aIle was about 30 times higher (70 ± 8.2 nM). In GPI preparations, depression of twitch was obtained in the micromolar range, i.e. at about 1000 times higher concentrations.

Similar results were obtained in binding studies using crude rat brain membranes. The order of affinity for the δ receptor, measured as inhibition constant (K_i) of [^{3}H]deltorphin I binding, was D-nLeu > D-Met > D-Ile > D-Val > D-aIle (see Table 1). However, the δ/μ selectivity obtained in these binding experiments was generally about one order of magnitude lower than the MVD/GPI potency ratio.

Binding studies were also performed using NG108-15 cells and CHO cells transfected with the δ receptor. [^{3}H]Deltorphin II bound to these cells was displaced by some of these peptides as shown in Table 2. The peptides tested displayed higher affinity for the δ receptor on NG108-15 cells than for the cloned receptor.

All the compounds had no affinity for the κ receptors ($K_i > 10$ μM, data not shown).

4. Discussion

The precursors of opioid peptides from Phyllomedusinae contain several copies of the end-products in their sequence. However, these are arranged in different orders. In case of the dermorphin precursor from *Ph. sauvagei*, the Met-deltorphin is first followed by four copies of dermorphin. Conversely, as shown in this communication, in *P. dacnicolor* the Ile-deltor-

Table 1
Pharmacological activities of Met-deltorphin and analogs

Peptide	IC_{50} (nM)			K_i (nM)		
	MVD	GPI	MVD/GPI	δ rec	μ rec	δ/μ
D-Met	2.5 ± 0.3	1700 ± 200	1.4×10^{-3}	9.6 ± 1.9	1630 ± 59	5.9×10^{-3}
D-aIle	70 ± 8.2	3200 ± 307	2.2×10^{-2}	54 ± 6.5	452 ± 68	1.2×10^{-1}
D-Ile	7.0 ± 0.9	4200 ± 375	1.7×10^{-3}	24 ± 3	1021 ± 157	2.3×10^{-2}
D-nLeu	0.76 ± 0.1	730 ± 63	1.0×10^{-3}	3.8 ± 0.8	314 ± 37	1.2×10^{-2}
D-Val	12.3 ± 1.5	750 ± 71	1.6×10^{-2}	34 ± 4.1	293 ± 35	1.1×10^{-1}

Peptides tested had the sequence Tyr-D-Xaa-Phe-His-Leu-Met-Asp-NH_2. The amino acid present in the second position is indicated in the first column. IC_{50}: agonist concentrations that produced 50% inhibition of the electrically evoked twitch; K_i: equilibrium dissociation constant of the competing ligand. The δ receptors were labeled with 0.3 nM [^{3}H]deltorphin I, the μ receptors with 0.5 nM [^{3}H]DAGO

Table 2
Inhibition constants of [^{3}H]deltorphin II (0.3 nM) binding to cells

Peptide	K_i (nM)	
	NG 108-15	CHO-d
D-Met	8.1 ± 0.7	17.8 ± 1.9
D-Ile	12.8 ± 1.5	43.2 ± 5.1
D-aIle	36.1 ± 4.2	118 ± 20

Met-deltorphin and two analogs with different residues at the second position were tested.

phin is last, near the carboxyl end. However, all these precursors are closely related to each other. In a phylogenetic tree constructed from the 3′-ends of these precursors, the relatedness mirrors the geographic distribution of the four species. *P. dacnicolor* from Southern Mexico and *A. annae* from Costa Rico form one closely related pair, *P. bicolor* from Amazonia and *P. sauvagei* from Argentina a second one.

Besides dermorphin, the two species investigated apparently contain only one additional opioid peptide which closely resembles Met-deltorphin. The natural compound which should contain D-aIle at the second position is a deltorphin. However, its affinity and selectivity for δ receptors is considerably lower than that of Met-deltorphin.

For comparison, we have also tested a number of related peptides which only differ from Met-deltorphin by the nature of the D-amino acid at position 2. Among these, the norleucine analog displays the highest affinity for δ receptors. Some of these peptides elicit unusual behavioral traits when injected into the brain of rats. These effects are currently under investigation.

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