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Identification and characterization of two dermorphins from skin extracts of the Amazonian frog *Phyllomedusa bicolor*

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Skin extracts of South American hylid frogs of the subfamily Phyllomedusinae contain dermorphins and deltorphins, opioid heptapeptides highly selective for either μ or δ receptors. In all these peptides, a D-amino acid is present in the second position. The structure of the precursors for Ala-deltorphins was recently deduced from cloned cDNAs derived from skin of *Phyllomedusa bicolor* (Richter et al. (1990) Proc. Natl. Acad. Sci. USA 87, 4836–4839). From the amino acid sequence of these precursors, the existence of three peptides related to dermorphin could be predicted. From methanol extracts of skin of *Ph. bicolor* we have isolated two of these peptides, [Lys^7]dermorphin-OH and [Trp^4,Asn^7]dermorphin-OH. The biological activity of these new dermorphins and their amidated counterparts is presented.

Amphibian skin; Dermorphin; Deltorphin; Opioid peptide

1. INTRODUCTION

Dermorphin and deltorphin are naturally occurring heptapeptides purified from skin extracts of the South American frog *Phyllomedusa sauvagei* [1,2] that show high affinity and selectivity for μ and δ opioid receptors, respectively. They share a similar N-terminal tripeptide sequence Tyr-D-Xaa-Phe, where D-Xaa is either D-Ala (dermorphin) or D-Met (deltorphin), and have an amidated COOH terminus. Using a cDNA library prepared from the skin of *Ph. sauvagei*, the amino acid sequence of several dermorphin precursors was deduced, one of which contained the genetic information also for deltorphin [3], now referred to as met-deltorphin. It was shown that the D-alanine or the D-methionine present in the final products were encoded by a normal codon for L-alanine or L-methionine, respectively. Two novel heptapeptides were subsequently isolated from skin extracts of the Amazonian frog *Phyllomedusa bicolor* that exhibited an even higher affinity for δ opioid receptors: they were named Ala-deltorphins [4].

Abbreviations: GPI, guinea-pig intestine; MVD, mouse vas deferens; DAGO, DAGMO, Tyr-D-Ala-Gly-Phe(Me)-Gly-ol

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The described peptides have the following sequences:

dermorphin
Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2

Met-deltorphin
Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2

Ala-deltorphin I
Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2

Ala-deltorphin II
Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2

Recently, the structure of four precursors for Ala-deltorphins I and II could be deduced from cloned cDNAs derived from the skin of *Ph. bicolor* [5]. These polypeptides contained one copy of Ala-deltorphin II, and either one, three or no copies of Ala-deltorphin I, respectively. In each case, a normal GCG codon for L-alanine was found in those positions where a D-alanine is present in the end product. Moreover, the cDNA sequences revealed that a glycine residue required for the formation of the COOH-terminal amide was present after the Ala-deltorphin sequences, as it was after the met-deltorphin and dermorphin sequences determined earlier [3]. From the amino acid sequences of the Ala-deltorphin precursors, the existence of three additional peptides related to dermorphin could be predicted. Due to the absence of glycine at the COOH terminus of these peptides, they should contain a free α-carboxyl group.

In this paper we describe the isolation of two of the three predicted peptides from methanol extracts of the skin of *Ph. bicolor*. The biological activity of these new

dermorphins and their amidated counterparts is presented.

2. MATERIALS AND METHODS

2.1. *Isolation of dermorphin-like peptides from* Ph. bicolor *skin*

Fresh skins (92 g) obtained from eight frogs captured near Oberá (Paraná, Brazil) were minced with scissors and extracted with 500 ml of methanol for 1 week at room temperature. The liquid was decanted and filtered, and the skin was extracted again with 5 parts (v/w) of 80% methanol. The filtrates were combined and stored in a dark bottle at 2°C until processed further. Most of this material was used earlier for the isolation and characterization of phyllomedusin [6], phyllocaerulein [7], phyllokinin [8] and Ala-deltorphins [4].

A volume of the filtered extracts corresponding to 2 g of fresh skin was lyophilized, redissolved in water, and then fractionated by HPLC on a Beckman model 332 system using a reverse-phase column (Aquapore RP-300, 7 × 250 mm, Brownlee Labs, Applied Biosystems) eluted with a 60-min gradient of 0–30% acetonitrile in 0.2% (by vol.) trifluoroacetic acid, at a flow of 2.0 ml/min. Elution of the peptides was monitored on a Beckman 160 spectrophotometer at 214 nm. In correspondence with each absorbance peak the effluent was collected in a tube and lyophilized. A 1% aliquot of the material from each tube was then used for assay of biological activity.

2.2. *Pharmacological assay on isolated tissue preparations*

The eluate of the HPLC column was tested for inhibitory action (IC_{50}, concentration producing 50% inhibition) on electrically evoked contractions in isolated preparations of myenteric plexus-longitudinal muscle obtained from the small intestine of guinea-pig (GPI) and preparations of vas deferens of mice (MVD) [9]. The synthetic reference peptide was dermorphin. The *Ph. bicolor* dermorphins were first tested on the MVD preparation, which is insensitive to contaminating stimulatory peptides, and subsequently on the guinea-pig ileum, which does not respond to deltorphins. To further characterize the frog dermorphins, bioassays were performed in the presence or absence of naloxone and the δ receptor antagonist naltrindole [10]. The GPI and MVD preparations were also used in parallel assays to test the activity of synthetic *Ph. bicolor* dermorphins (both deamidated and amidated forms), dermorphin and some dermorphin-like peptides as well as other opiate compounds (see Table II).

2.3. *Structural analysis*

Amino acid analyses were performed with a Pharmacia Alpha Plus 4151 analyzer after vapor phase hydrolysis of the peptides (1–2 nmol) in 6 N HCl for 24 h. Peptide sequences were determined by automated Edman degradation with an Applied Biosystems model 475A gas-phase sequencer. The presence of a D-amino acid in the sequence was confirmed by reaction with D-amino acid oxidase (Sigma) as reported [4]. The presence of a free α-carboxyl group was determined from the electrophoretic mobility of the peptides at pH 6.5 according to Offord [11] and by mass spectrometry.

2.4. *Peptide synthesis*

Peptides were prepared by solid-phase synthesis on a Pharmacia Biolynx automated peptide synthesizer according to fluoren-9-ylmethoxycarbonyl (Fmoc)-polyamide active ester chemistry [12]. The products were purified by reverse-phase HPLC and the expected amino acid sequences were confirmed by automated Edman degradation.

3. RESULTS AND DISCUSSION

The elution profile from the HPLC column of the peptides from the skin extract of *Ph. bicolor* is shown in Fig. 1. Each fraction was tested for biological activity

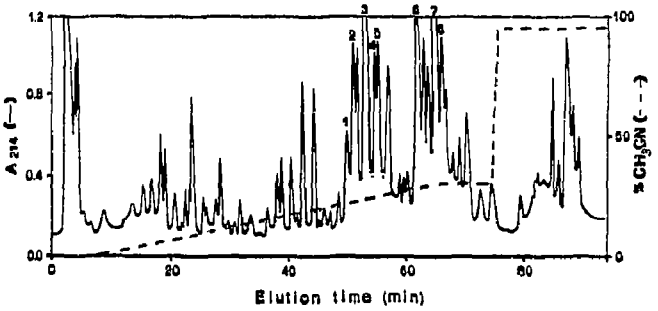

Fig. 1. Reverse-phase HPLC of a methanol extract from the skin of *Ph. bicolor*. Peaks 1 through 8 were analyzed further (see Table I).

on the guinea-pig ileum and the mouse vas deferens. The dermorphin-like activity was present in peaks 1 and 8, while deltorphin-like activity was found in peaks 4 and 5 (see Fig. 1). In peaks 2, 3 and 6 a stimulatory activity on guinea-pig ileum was detected. From the amino acid composition of these fractions (see Table I) it was obvious that peak 2 corresponds to phyllomedusin [6], peak 3 to phyllocaerulein [7], peak 4 to Ala-deltorphin II, peak 5 to Ala-deltorphin I [4], and peak 6 to phyllokinin [8].

The amino acid sequences of the dermorphin-like peptides present in peaks 1 and 8 were determined. These were found to be Tyr-Ala-Phe-Gly-Tyr-Pro-Lys (peak 1) and Tyr-Ala-Phe-Trp-Tyr-Pro-Asn (peak 8). The electrophoretic mobility of both peptides was consistent with the presence of a free α-carboxyl group. This result was also confirmed by fast-atom-bombardment mass spectral analysis (*m/z* 845 and 960, respectively, for the two peptides). Comparison of amino acid analyses performed on acid hydrolysates of the peptides before and after treatment with D-amino acid oxidase confirmed the presence of a D-alanine in both peptides. Consequently, the two novel peptides were named [Lys^7]dermorphin-OH and [Trp^4,Asn^7]dermorphin-OH, respectively.

In the extract from the skin of *Ph. bicolor* the dermorphin pentapeptide Tyr-D-Ala-Phe-Trp-Asn-OH, also predicted from the sequence of the cloned cDNAs, could not be detected as a distinct chromatographic peak or by bioassay. This may be due to the low content of this peptide as well as to its relatively low biological activity (see below).

The peptide present in peak 7 was devoid of any biological activity on isolated smooth muscle preparations. Its amino acid composition (see Table I) does not correspond to any known peptide. As automated Edman degradation gave no results, the peptide was treated with 1 N HCl in methanol at 22°C for 24 h [13] to hydrolyze pyroglutamyl residues. After this treatment, the following sequence could be obtained: Glp-Glu-Lys-Pro-Phe-Tyr-Pro-Pro-Pro-Ile-Tyr-Pro-Val. The electrophoretic mobility of the peptide and the result of mass spectral analysis (*m/z* 1557) was consistent

Table I

Amino acid composition of peptides purified from *Ph. bicolor* skin extract

Amino acid	Peptide							
	1	2	3	4	5	6	7	8
Aspartic acid		2.0 (2)	1.1 (1)		0.8 (1)			1.1 (1)
Serine						1.0 (1)	1.0 (1)	
Glutamic acid		1.0 (1)	2.0 (2)	0.9 (1)			2.4 (2)	
Proline	1.5 (1)	1.1 (1)				2.7 (3)	5.2 (5)	1.2 (1)
Glycine	1.6 (1)	1.2 (1)	1.0 (1)	0.8 (1)	0.8 (1)	1.2 (1)	1.3 (1)	0.9 (1)
Alanine	1.5 (1)			1.0 (1)	0.9 (1)			
Valine				1.9 (2)	1.8 (2)			
Methionine		0.4 (1)	0.9 (1)					
Isoleucine		0.8 (1)				1.1 (1)	1.2 (1)	
Leucine		1.0 (1)						
Tyrosine	2.9 (2)		1.0 (1)	0.9 (1)	0.8 (1)	0.9 (1)	0.9 (1)	1.8 (2)
Phenylalanine	1.8 (1)	1.0 (1)	1.1 (1)	1.0 (1)	0.9 (1)	2.1 (2)	1.2 (1)	1.2 (1)
Lysine	1.4 (1)						1.1 (1)	
Arginine		0.8 (1)				1.8 (2)		
Tryptophan			++ (1)					++ (1)

The composition from sequence analysis of each peptide is indicated by the numbers in parentheses. Presence of tryptophan was indicated by absorbance at 280 nm and Ehrlich reaction. Peptide numbering refers to the peaks in Fig. 1. (1) [Lys^7]dermorphin-OH; (2) phyllomedusin [7]; (3) phyllocaerulein [8]; (4) Ala-deltorphin II [4]; (5) Ala-deltorphin I [4]; (6) [Hyp^3,Ser^6]phyllokinin [9]; (7) tryptophyllin-like peptide; (8) [Trp^4,Asn^7]dermorphin-OH.

with the presence of a free α-carboxyl group. The sequence of this peptide is similar to that reported by Montecucchi et al. [14] for a tridecapeptide isolated from *Ph. rohdei* skin extracts, belonging to the class of the so- called tryptophyllins. However, in the peptide from *Ph. bicolor*, besides two additional substitutions, the tryptophan residue in position 5 was replaced by a tyrosine.

The results of parallel bioassays of the natural dermorphins and a number of synthetic analogs are presented in Table II. In each case, the peptides with a free α-carboxyl group at the COOH terminus and the corresponding amides were tested. For comparison, the results obtained with dermorphin [1–5], DAGO (DAGMO: Tyr-D-Ala-Gly-Phe(Me)-Gly-ol) and morphine were included in the table.

Table II

Inhibitory potency (IC_{50}) of natural dermorphins, their amidated forms, DAGO and morphine on electrically evoked contractions of mouse vas deferens (MVD) and guinea-pig ileum (GPI) preparations

Peptide	IC_{50}, nM [mean ± S.E.M. (*n*)]		MVD/GPI
	MVD	GPI	
Dermorphin (Derm)	16.5 ± 1.8 (68)	1.29 ± 0.09 (8)	12.8
Derm-OH	28.1 ± 2.4 (5)	4.5 ± 0.32 (5)	6.24
[Hyp^6]Derm	18.1 ± 2.9 (5)	1.6 ± 0.12 (4)	11.3
[Hyp^6]Derm-OH	33.1 ± 2.0 (4)	5.16 ± 0.4 (4)	6.4
[Lys^7]Derm	13.6 ± 1.5 (22)	1.15 ± 0.13 (22)	11.8
[Lys^7]Derm-OH	56.3 ± 7.8 (15)	3.8 ± 0.45 (10)	14.7
[Trp^4,Asn^7]Derm	6.6 ± 0.9 (23)	0.58 ± 0.06 (18)	11.4
[Trp^4,Asn^7]Derm-OH	10.4 ± 1.3 (11)	1.3 ± 0.2 (10)	8.0
[Trp^4,Asn^5]Derm[1–5]	73.7 ± 9.1 (10)	5.0 ± 0.52 (10)	14.6
[Trp^4,Asn^5]Derm[1–5]OH	205.2 ± 25.4 (13)	13.1 ± 1.2 (10)	15.6
Derm[1–5]	21.4 ± 3.1 (10)	2.47 ± 0.3 (9)	8.7
DAGO	115.0 ± 21.0 (10)	7.1 ± 0.9 (10)	16.2
Morphine	793.0 ± 97.0 (15)	51.6 ± 7.3 (12)	15.4

In parentheses is the number of experiments

From these data the following conclusions can be drawn:

(a) dermorphin, [Hyp^6]dermorphin and [Lys^7]dermorphin are approximately equiactive both on MVD and GPI preparations;

(b) [Trp^4, Asn^7]dermorphin is 3 times more potent than the other three dermorphins;

(c) the dermorphin pentapeptide [Trp^4, Asn^5] is 8 to 11 times less potent than the corresponding heptapeptide [Trp^4, Asn^7] and is also less potent than dermorphin[1–5];

(d) the deamidated peptides are 2 to 4 times less potent than the corresponding amidated forms. Deamidation may produce a very slight shift of selectivity towards δ receptors (decreased MVD/GPI ratio).

(e) DAGO was 5–6 times less potent than dermorphin and morphine 40 times less potent, with similar MVD/GPI ratios.

It should be noted that the preservation of the activity and selectivity towards μ receptors of dermorphins is not significantly compromised by either deamidation or shortening of the peptide chain. Conversely, the affinity of Ala-deltorphins for δ receptors is drastically reduced by these changes, each causing at least a 30-fold decrease. For example, Ala-deltorphin I [1–5]-OH is about 3000 times less potent than the corresponding amidated heptapeptide [15].

On the basis of amino acid analysis and bioassay we

estimate that one gram of fresh skin of *Ph. bicolor* contains about 25 μg of [Lys^7]dermorphin-OH and 68 μg of [Trp^4, Asn^7] dermorphin-OH. The total amount of dermorphins present in the skin of *Ph. bicolor* is thus similar to that found in other Phyllomedusinae [16].

It is finally worth mentioning that in radioimmunoassays with an anti-dermorphin serum, skin extracts from *Ph. bicolor* gave completely negative results (unpublished observations). Obviously, this antiserum did not cross-react with the *Ph. bicolor* dermorphins, and this retarded the identification of these novel peptides.

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