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cDNAs encoding [D-Ala^2]deltorphin precursors from skin of *Phyllomedusa bicolor* also contain genetic information for three dermorphin-related opioid peptides

(amphibian skin peptides/precursors)

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ABSTRACT **We present the structure of four precursors for [D-Ala^2]deltorphins I and II as deduced from cDNAs cloned from skin of the frog *Phyllomedusa bicolor*. These contain the genetic information for one copy of [D-Ala^2]deltorphin II and zero, one, or three copies of [D-Ala^2]deltorphin I. In each case, the D-alanine of the end product is encoded by a normal GCG codon for L-alanine. In addition, the existence of three peptides related to dermorphin was predicted from the amino acid sequence of the precursors. These peptides were synthesized with a D-alanine in position 2 and their pharmacological properties were tested. Two of them, [Lys^7]dermorphin-OH and [Trp^4,Asn^7]dermorphin-OH, were found to have roughly the same affinity and selectivity for μ-type opioid receptors as dermorphin.**

A group of opioid peptides has been discovered in the skin of South American frogs belonging to the subfamily Phyllomedusinae. These are heptapeptides with the common amino-terminal sequence Tyr-D-Xaa-Phe, in which D-Xaa is either D-alanine or D-methionine. The first peptide isolated from several species of these frogs was dermorphin, which was shown to have high affinity and selectivity for μ-type opioid receptors (1, 2). By using a cDNA library prepared from skin of *Phyllomedusa sauvagei*, the amino acid sequence of several dermorphin precursors was established (3) and it was shown that the D-alanine present in the final product was encoded by a normal codon for L-alanine. From the sequence of one of these cDNAs, the existence of another peptide was predicted that contained methionine as the second amino acid (3). This peptide with a D-methionine as the second amino acid was subsequently isolated from skin of *Ph. sauvagei* (4, 5). As demonstrated independently by three groups (4, 6, 7), this peptide had a higher affinity and selectivity for δ opioid receptors than any other known natural compound. We have therefore proposed the name deltorphin for this compound (4). Subsequently, two additional peptides with even higher affinity for the δ receptor were isolated from the skin of *Phyllomedusa bicolor* (8). Like dermorphin, these peptides contain D-alanine as the second amino acid and they have been termed [D-Ala^2]deltorphins I and II. A comparison of the sequence of these peptides is shown in Table 1.

In this communication we present the structure of four precursors for [D-Ala^2]deltorphins I and II as deduced from cDNAs cloned from skin of *Ph. bicolor*. From the sequence of these cDNAs,§ the existence of three additional peptides related to dermorphin was predicted (Table 1). The receptor-binding characteristics of these peptides are presented.

MATERIALS AND METHODS

mRNA Preparation and cDNA Cloning. Two live specimens of *Ph. bicolor* were obtained from Manaus (Brazil). Total poly(A)-rich RNA was prepared from their skin as described previously for *Ph. sauvagei* (3), transcribed into double-stranded cDNA by Moloney murine leukemia virus reverse transcriptase (BRL), and cloned by a standard procedure (9) into the *Pst* I site of the pBluescript vector (Stratagene) by G·C-tailing. About 22,000 clones were first screened with the oligonucleotide d(ACNACYTCRAANGCRTA), where Y stands for C and T, R stands for A and G, N stands for all four deoxynucleotides. This oligonucleotide is complementary to the codons for the amino-terminal sequence Tyr-Ala-Phe-Glu-Val-Va(l) of [D-Ala^2]deltorphin II. For the screening procedure, filter replicas were made on nitrocellulose (Schleicher & Schüll BA85) and hybridized with the labeled oligonucleotide at 40°C as described by Singer-Sam *et al.* (10). This hybridization temperature was 8°C below the minimal melting temperature (10). Filters were then rescreened with the same oligonucleotide but containing A and G in the sixth position. This probe hybridizes to the codons of [D-Ala^2]deltorphin I, which has aspartic acid as the fourth amino acid. Several clones that gave a positive signal with both probes were selected, and DNA was isolated by standard procedures and sequenced by the chemical degradation and the enzymatic method (11, 12). After sequencing confirmed AD2 as a cDNA clone coding for a [D-Ala^2]deltorphin precursor, it was used to rescreen the entire cDNA library. Two dozen clones that showed strong hybridization signals were isolated and at least partly characterized by cleavage with restriction enzymes and sequencing. This provided evidence for the existence of at least four types of precursors.

Synthesis of Peptides. Three peptides predicted from the sequence of the two deltorphin precursors were synthesized using fluorenylmethoxycarbonyl–polyamide active-ester chemistry on a Biolynx automated peptide synthesizer (Pharmacia Biochrom, Cambridge, U.K.). The products were purified by HPLC as described (8). The structure of the peptides was confirmed by amino acid analysis and automated Edman degradation.

Biological Activity of Synthetic Peptides. The synthetic peptides were tested for their inhibitory action on electrically evoked contractions in isolated preparations of myenteric plexus-longitudinal muscle obtained from the small intestine of male guinea pigs and the vasa deferentia of mice (4, 8).

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34560).

Table 1. Primary structure of opioid peptides from skin of Phyllomedusinae

Peptide	Sequence	Ref.
Deltorphin	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	3
[D-Ala^2]Deltorphin I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	8
[D-Ala^2]Deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	8
Dermorphin	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	1
"New peptides"	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-OH	
	Tyr-D-Ala-Phe-Trp-Tyr-Pro-Asn-OH	
	Tyr-D-Ala-Phe-Trp-Asn-OH	

Dermorphin and deltorphin are present in skin of *Ph. sauvagei*; the [D-Ala^2]deltorphins were isolated from *Ph. bicolor*. The "new peptides" (without amidation at the carboxyl terminus) were predicted from the cDNA sequence of the [D-Ala^2]deltorphin precursors as described in this communication.

Binding Assays. Binding of peptides to μ and δ sites was assayed in crude membrane preparations from rat brain at pH 7.4 in 50 mM Tris·HCl buffer. Each assay contained, in a final volume of 2 ml, the membrane preparation (0.6–0.8 mg of protein, equivalent to 20–27 mg of brain tissue) and the tritiated ligand (Radiochemical Centre) with or without unlabeled ligand. For μ binding sites, tritiated [2-(D-alanine), 4-(*N*-methyl)phenylalanine,5-glycinol]enkephalin ([H^3]-DAGO) was used, while the δ binding sites were labeled with tritiated [2-(D-penicillamine),5-(D-penicillamine)]enkephalin ([H^3]DPDPE). Nonspecific binding of radioactivity was determined in the presence of 1 μM DAGO and 1 μM DPDPE in μ and δ binding assays, respectively.

RESULTS

A cDNA library from skin of *Ph. bicolor* was constructed and about 22,000 clones were sequentially screened with two oligodeoxynucleotides complementary to the codons for the sequence Tyr-Ala-Phe-Asp(or Glu)-Val-Va(l). Clones hybridizing with both probes were isolated and those with the largest inserts were investigated further. Four different types of cDNAs encoding [D-Ala^2]deltorphin precursors were found. The nucleotide sequence of clone AD2 is shown in Fig. 1. It has an insert of 781 base pairs excluding the poly(A) tail. The single open reading frame can be translated into a precursor polypeptide comprising 227 amino acids that starts

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...TCAGTACTTCCTGAATAACAAGACCCAACATGTCTTTCTTGAAGAAATCGCTTCTGCTTGTA
                                    M  S  F  L  K  K  S  L  L  L  V

CTTTTCCTTGGATTAGTGTCCCATTCCGTTTGTAAAGAAGAGAAAAGAGAGACTGAAGAGGAG
 L  F  L  G  L  V  S  H  S  V  C  K  E  E  K  R  E  T  E  E  E

AATGAAAATGAGGAAGAAAATCATGAAGTGGGAAGTGAGATGAAGAGATATGCGTTCTGGTAT
 N  E  N  E  E  E  N  H  E  V  G  S  E  M  K  R  TyrAlaPheTrpTyr

CCGAATAGAGACACTGAAGAGAAGAATGAAAATGAGGAAGAAAATCAGGAAGAGGGAAGTGAG
ProAsn R  D  T  E  E  K  N  E  N  E  E  E  N  Q  E  E  G  S  E

ATGAAGAGATATGCGTTCGGCTATCCGAAAAGAGAGCCTGAAGAGGAAAATGAGAATGAGGAA
 M  K  R TyrAlaPheGlyTyrProLys R  E  P  E  E  E  N  E  N  E  E

GAAAATCATGAAGAGGGAAGTGAGATGAAGAGATATGCGTTTGAAGTTGTGGGAGGAGAAGCT
 E  N  H  E  E  G  S  E  M  K  R TyrAlaPheGluValValGly G  E  A
          §
AAGAAAATGAAAAGAGAACCTGAAGAGGAAAATGAGAATGAGGAAGAAAATCATGAAGAGGGA
 K  K  M  K  R  E  P  E  E  E  N  E  N  E  E  E  N  H  E  E  G
                                                        #
AGTGAGATGAAGAGATATGCGTTTGACGTTGTGGGAGGAGAAGCTAAGAAAATGAAAAGAGAG
 S  E  M  K  R TyrAlaPheAspValValGly G  E  A  K  K  M  K  R  E

CCTGAAGAGGAAAATGAGAATGAGGAAGAAAATCATGAAGAGGGAAGTGAGATGAAGAGATAT
 P  E  E  E  N  E  N  E  E  E  N  H  E  E  G  S  E  M  K  R Tyr

GCGTTTGACGTTGTGGGAGGAGAAGCTAAGAAAATGAAAAGAGAGCCTGAAGAGGAAAATGAG
AlaPheAspValValGly G  E  A  K  K  M  K  R  E  P  E  E  E  N  E

AATGAGGAAGAAAATCATGAAGAGGGAAGTGAGATGAAGAGATATGCGTTTGACGTTGTGGGA
 N  E  E  E  N  H  E  E  G  S  E  M  K  R TyrAlaPheAspValValGly
                §
GGAGAAGCTAAGAAAATGTAATATTTCATAACTTAAAGGAGCACAATTATCAGTTATATGCCA
 G  E  A  K  K  M  /

AACATATATTAAATGATAGATAACTT(polyA)
```

FIG. 1. Nucleotide and deduced amino acid sequences of clone AD2 from skin of *Ph. bicolor*. The amino acid sequence is given in single letters; only the deltorphin copies and the sequences of predicted peptides related to dermorphin are emphasized by using the three-letter code. The nucleotide sequence of clone AD3 was almost identical to that of AD2 except for a deletion of two codons (overlined) and a silent point mutation (not shown). AD3 also had a somewhat longer 5′ untranslated end than AD2 (not shown). In clone AD7 the region between the marks (§) was absent. Clone AD8 had a smaller deletion starting at the nucleotide marked # and ending, as in AD7, just before the stop codon.

Table 2. Inhibitory potency (IC_{50}) of dermorphin, [Lys^7]dermorphin-OH, [Trp^4,Asn^7]dermorphin-OH, and Tyr-D-Ala-Phe-Trp-Asn-OH (pentapeptide) on electrically evoked contractions of guinea pig ileum (GPI) and mouse vas deferens (MVD) preparations

Peptide	IC_{50}, nM		MVD/GPI
	MVD	GPI	
Dermorphin	17.8 ± 2.1 (29)	1.06 ± 0.06 (14)	16.8
[Lys^7]Dermorphin	52 ± 4.7 (10)	1.72 ± 0.08 (7)	30.2
[Trp^4,Asn^7]Dermorphin	7.4 ± 1.3 (13)	1.30 ± 0.08 (15)	5.7
Pentapeptide	88 ± 5.3 (15)	11.3 ± 0.4 (11)	7.8

Values are means ± SEM; the number of experiments is given in parentheses.

with an initiating methionine and a typical signal peptide. In the sequence encoding the propolypeptide, four copies of a 108-base repeat and two shorter variants thereof are present (Fig. 1). Of the former repeats, three contain the sequence of [D-Ala^2]deltorphin I and the other that of [D-Ala^2]deltorphin II. The two variants encode peptides similar to dermorphin. All these known and predicted peptides contain the amino-terminal sequence Tyr-Ala-Phe, which is preceded by the typical prohormone processing signal Lys-Arg. Following the deltorphin sequence is the more complex processing sequence Gly-Glu-Ala-Lys-Lys, the glycine being required for the formation of the carboxyl-terminal amide (13, 14). The same flanking sequences have also been found in the dermorphin precursors from *Ph. sauvagei* (3). Conversely, after the dermorphin variant that contains tryptophan, a single arginine residue is present in the [D-Ala^2]deltorphin precursor. Processing at single arginines has been observed in several prohormones (15) as well as in the precursors of peptides present in the skin of the frog *Xenopus laevis* (16, 17). The other dermorphin relative terminates with the sequence Pro-Lys-Arg. Following endoproteolytic cleavage after the arginine residue, the carboxypeptidase can be expected to hydrolyze the Lys-Arg bond but not the Pro-Lys bond (15).

The nucleotide sequence of a second clone, termed AD3, was almost identical to that of AD2 (Fig. 1), the main difference being a deletion of two codons in the sequence of one of the dermorphin homologues. The final product in this instance would thus be a pentapeptide rather than a heptapeptide.

The two other cloned cDNAs, AD7 and AD8, were identical to AD2 in the coding and 3′ regions, and both terminated with a poly(A) tract. However, these cDNAs have a large deletion in the central part and contain the genetic information for only one copy of [D-Ala^2]deltorphin II (AD7) or one copy each of [D-Ala^2]deltorphin II and I (AD8). It is not known whether the corresponding mRNAs are transcribed from separate genes or formed through alternative splicing of the primary transcript of the AD2 gene. By Northern blot analysis of poly(A)$^+$ RNA from skin of *Ph. bicolor*, only a broad band in the range of 500–900 nucleotides was observed (data not shown).

From the sequence of these precursor polypeptides, the existence of three additional opioid peptides in the skin of *Ph. bicolor* was predicted (Table 1). While the deltorphin sequences are all followed by a glycine residue required for the formation of terminal amides, this is not the case for the dermorphin-like peptides. These peptides should thus contain a free α-carboxyl group.

The three peptides predicted on the basis of the cDNA are [Lys^7]dermorphin-OH, [Trp^4,Asn^7]dermorphin-OH, and the pentapeptide lacking residues 5 and 6 of the latter (Table 1). These peptides with a D-alanine in position 2 were synthesized by solid-phase methods and purified by HPLC, and their biological activity was compared to that of dermorphin. The two heptapeptides inhibited the electrically evoked contraction of the guinea pig ileum about equally as well as dermorphin (Table 2). Also, their selectivity for μ versus δ receptors was comparable to that of dermorphin. The pentapeptide was about 1/10th as active as dermorphin. Receptor binding assays with rat brain preparations demonstrated that [Trp^4,Asn^7]dermorphin had a comparable affinity and an even somewhat higher selectivity for μ receptors than dermorphin, whereas [Lys^7]dermorphin and the pentapeptide had a somewhat lower affinity (Table 3).

In the sequence of these precursors, the segments encoding deltorphin and dermorphin and the respective processing sites are separated by highly conserved spacer peptides. Processing at Lys-Arg pairs will also liberate these spacer peptides containing 19 amino acids, of which 11 are glutamic residues. Skin secretion of these frogs should thus contain high amounts of these peptides, but it is not known whether they have any biological function. However, it is noteworthy that the sequence of these spacer peptides is highly conserved between *Ph. bicolor* and *Ph. sauvagei* (Fig. 2).

DISCUSSION

The sequence of four very similar [D-Ala^2]deltorphin precursors was deduced from cloned skin cDNAs of *Ph. bicolor*. They all contain the genetic information for one copy of [D-Ala^2]deltorphin II, for up to three copies of [D-Ala^2]deltorphin I, and for two peptides related to dermorphin. It is not known how many genes for these precursors exist in the genome of *Ph. bicolor*, nor whether this frog is tetraploid like the related species *Ph. burmesteri* (18).

Formerly, the existence of deltorphin was predicted from a cDNA sequence of the dermorphin precursor from skin of *Ph. sauvagei* (3), and this peptide was subsequently detected in skin extracts (4). We likewise expect the peptides predicted from the sequence of the [D-Ala^2]deltorphin precursors to be present *in vivo*. Unlike dermorphin and the deltorphins, however, these peptides should exist with a free α-carboxyl rather than an amide at the carboxyl terminus. Two of these predicted peptides, [Lys^7]dermorphin-OH and [Trp^4,Asn^7]dermorphin-OH, were found to have a similar affinity and selectivity for the μ opioid receptor as dermorphin. A comparison of the various peptides (Table 1) as well as earlier studies show that the amino-terminal tripeptide is essential for receptor binding, that the fourth residue apparently has little influence, and that the last three amino acids are important for receptor selectivity. A study using different

Table 3. Receptor binding affinity (K_i) and receptor selectivity of dermorphin, [Lys^7]dermorphin, [Trp^4,Asn^7]dermorphin, and the pentapeptide Tyr-D-Ala-Phe-Trp-Asn-OH

Peptide	K_i, nM (mean ± SEM)		Selectivity ($K_i\delta/K_i\mu$)
	μ	δ	
Dermorphin	1.19 ± 0.03	150 ± 12	126
[Lys^7]Dermorphin	8.41 ± 1.2	255 ± 17	30
[Trp^4,Asn^7]Dermorphin	2.18 ± 1.2	780 ± 57	358
Pentapeptide	3.22 ± 1.6	1200 ± 75	373

a) GAGAGTGAAGAGGAGAATGAAAATGAAGAA***AATCATGAAGAGGGAAGTGAGATG

b) GAGCCTGAAGAGGAAAATGAGAATGAGGAAGAAAATCATGAAGAGGGAAGTGAGATG

a) GluSerGluGluGluAsnGluAsnGluGlu***AsnHisGluGluGlySerGluMet

b) GluProGluGluGluAsnGluAsnGluGluGluAsnHisGluGluGlySerGluMet

FIG. 2. Comparison of nucleotide and amino acid sequences of "spacer regions" from the dermorphin precursor of *Ph. sauvagei* (ref. 3) (*a*) and the [D-Ala^2]deltorphin precursors of *Ph. bicolor* (*b*). In prodermorphin, two repeats differing from each other by two amino acids are present (3). Only the one with the higher homology to the spacer of the [D-Ala^2]deltorphin precursors is shown. The asterisks mark a deletion that was introduced to maximize the homology. Differences are underlined.

synthetic dermorphin/deltorphin hybrid peptides has confirmed this notion (19).

Following its discovery in 1981, dermorphin and a variant form containing hydroxyproline instead of proline were thought to be the only opioid peptides present in amphibian skin. Through a combination of cDNA cloning, pharmacological testing, and peptide chemistry, several additional peptides with this activity have been discovered. Two subgroups can now be distinguished based on the binding to different opioid receptors. Dermorphin and its relatives have a high affinity and selectivity for μ receptors, whereas the deltorphins are specific δ-receptor agonists. All these peptides have the amino-terminal sequence Tyr-D-Xaa-Phe in common, where Xaa is in all but one case D-alanine. Since detailed studies have been restricted to only two species of Phyllomedusinae, *Ph. sauvagei* and *Ph. bicolor*, the presence of yet additional peptides of this type in other species is likely.

The evolutionary aspects of the genes for these precursors are of some interest. The cDNA and the predicted amino acid sequence of the dermorphin precursor from *Ph. sauvagei* (3) and the [D-Ala^2]deltorphin precursor from *Ph. bicolor* show a high degree of homology over most parts. A comparison of spacer regions of the two precursors, which are located between the copies of the end products (Fig. 2), reveals that these sequences are 86% and 89% identical at the cDNA and amino acid level, respectively. This overall homology is interrupted by mutational "hot spots" in the regions encoding the final products. For example, dermorphin and deltorphin differ from each other by 13 point mutations, and these in turn differ from the [D-Ala^2]deltorphins by 9 mutations each. For a sequence of only seven codons, this indicates a high rate of mutational change. Comparisons of homologous precursors from different species usually reveal the greatest similarities for the segments encoding the mature product and more variations in those parts belonging to the proregions. The conserved A and B chains of insulin as opposed to the highly variable C peptide illustrate this point. It is not known why the opposite situation applies with the dermorphin and deltorphin precursors from two frog species of the same subfamily. One possibility is that these spacer sequences were conserved because they have some important steric function in, for example, the biosynthesis of the D amino acid. Alternatively, these highly acidic peptides may well have biological activity important for the function of the skin secretion of these tree frogs.

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