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Isolation of dermenkephalin from amphibian skin, a high-affinity δ-selective opioid heptapeptide containing a D-amino acid residue

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The predicted amino acid sequence of the biosynthetic precursor of dermorphin, a highly potent and nearly specific μ-opioid peptide from amphibian skin, contains four repeats of the dermorphin progenitor sequence and one single copy of a different heptapeptide sequence. We have developed a specific enzyme immunoassay and used synthetic peptides to detect and purify the new predicted heptapeptide (2.4 μg/g dry skin) from the skin of the *Phyllomedusa sauvagei* frog from which dermorphin was originally isolated. The identity of the novel pro-dermorphin related peptide, Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2, was established by co-chromatography with synthetic peptides on reverse-phase HPLC, immunological analysis, gas-phase sequencing, mass spectrometry and by pharmacological assays. Opioid-binding assays in vitro demonstrated that both the natural and synthetic heptapeptides displayed exceptionally high selectivity and affinity towards the δ-opioid receptors. Because of its origin and its δ-opioid (enkephalin) activity and specificity, this novel D-amino acid containing peptide is named dermenkephalin.

Opioid receptor, δ-; Amino acid containing peptide, D-; Dermorphin; Dermenkephalin

1. INTRODUCTION

The dermatous glands of amphibians synthesize and expel a rich variety of biologically active peptides [1,2]. Among secretory peptides isolated from amphibian skin, dermorphin [3] – Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 – is the most selective high-affinity endogenous μ-opioid agonist known to date [3–9]. With a cDNA library prepared from the skin of the South American frog *Phyllomedusa sauvagei*, the primary structure of the dermorphin biosynthetic precursor was elucidated recently [10]. The precursor (fig.1) was shown to code for a repetitive pattern of five extensively homologous sequences of about 35 amino acids, four of which code for dermorphin while the fifth codes for a different heptapeptide sequence flanked by the same processing signals as those flanking the dermorphin progenitor sequences. Assuming the same processing cascade involved as for the release of dermorphin, processing of pro-dermorphin is expected to yield, beside four copies of dermorphin, one single copy of a new C-terminally amidated heptapeptide containing a D-amino acid residue at the second position i.e., Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2.

Using synthetic peptides in biological and pharmacological assays, we have already shown [11] that this putative new heptapeptide, named der-

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Abbreviations: DREK, dermenkephalin; DRM, dermorphin; EIA, enzyme immunoassay; BSA, bovine serum albumin; RP-HPLC, reverse-phase high-performance liquid chromatography; DTLET, [D-Thr^2,Leu^5]-enkephalyl-Thr^6; NHS, *N*-hydroxysuccinimide; DCC, *N,N'*-dicyclohexylcarbodiimide

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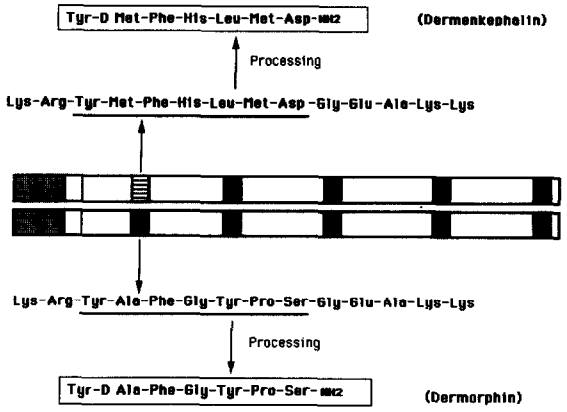

Fig.1. Schematic representation of the structural organisation of the dermorphin biosynthetic precursors from the frog *Phyllomedusa sauvagei* [10]. The N-terminal dashed sequence is the predicted signal peptide sequence. Positions of dermorphin progenitor sequence, Tyr-Ala-Phe-Gly-Tyr-Pro-Ser, which is flanked by Lys-Arg at the amino end and by Gly-Glu-Ala-Lys-Lys at the carboxyl end, are shaded. One of the precursors (upper representation) has a dermorphin copy replaced with a distinct progenitor sequence, Tyr-Met-Phe-His-Leu-Met-Asp, whose processing yields dermenkephalin, i.e. Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2.

menkephalin, acts as a highly potent and fully selective agonist for the δ-opioid receptor. In this study, we report on the isolation and purification of dermenkephalin from the skin of *Phyllomedusa sauvagei* as characterized by its chemical structure and pharmacological properties.

2. MATERIALS AND METHODS

2.1. *Peptide synthesis*

Dermenkephalin and analogs were prepared in our laboratory as described previously [11,12] by stepwise manual solid-phase synthesis using the preformed symmetric anhydride technique. Homogeneity of synthetic products was assessed by analytical RP-HPLC, thin-layer chromatography, amino acid analysis and fast atomic bombardment mass spectrometry as described [12].

2.2. *Immunogen preparation and immunization*

The side chain carboxyl of aspartate at the C-terminal end of DREK was coupled to BSA for immunogen preparation via amide bond, with prior formation of NHS ester [13]. To 100 μl DREK (10 μmol) were successively added 100 μl NHS (100 μmol) and 100 μl DCC (10 μmol) all in anhydrous dimethylformamide (DMF). The mixture was left to react in the dark at 22°C for 18 h under continuous stirring, then added to 7 ml (21 mg) BSA in 0.1 M borate buffer, pH 9, and 30 min later, the reaction mixture was dialysed against NaCl (0.9%). About 1 mg of immunogen (DREK-BSA conjugate) in 1 ml NaCl (0.9%) was emulsified with 1 ml Freund's complete adjuvant (Difco Lab., Detroit, MI). The emulsion was injected intradermally into the flank area of 3 New Zealand white rabbits at multiple sites (30–40 points). Rabbits were boosted 3 times at 6 week intervals then bled ten days after each boosting. Sera were screened for antibodies using the enzyme immunoassay procedure. The bleedings collected after the fourth booster were selected to obtain antisera for routine EIA.

2.3. *Preparation of the enzymatic tracer and the EIA procedure*

DREK was coupled to acetylcholinesterase (AChE; EC 3.1.1.7) from the electric organ of electric eel *Electrophorus electricus* as previously described for DRM [14]. The enzymatic tracer was either stored at −80°C or freeze dried and kept at 4°C until use. Solid-phase EIA was performed with Titertek microtitration equipment (Flow Laboratories, Finland) which included an automatic plate washer (Microplate Washer 120), an automatic plate dispenser (Autodrop) and an automatic plate reader (Multiskan MC). All assays were performed in the following EIA buffer: 0.1 M phosphate buffer, pH 7.4, containing 0.4 M NaCl, 1 mM EDTA, 0.1% BSA and 0.01% sodium azide. The detailed EIA procedure was described elsewhere [15]. In short, 96 well microtiter plates (Nunc 96F, Denmark) were coated with mouse monoclonal antibodies specific to rabbit IgG in order to ensure separation between bound and free moieties of the tracer [16]. Before use, the plates were washed with 10 mM phosphate buffer, pH 7.4, in the presence of 0.05% Tween 20. Reagents were dispensed in the following order: 50 μl standard or biological extract, 50 μl DREK-AChE conjugate and 50 μl DREK antiserum 1/10000 all diluted in EIA buffer. The plates were left at 4°C for 18 h and washed again as above, then 200 μl of Ellman's medium [17] were dispensed into each well. The reaction was allowed to take place for 1 h and the absorbance measured at 414 nm with a Multiskan MC spectrophotometer.

2.4. *Characteristics of the dermenkephalin enzyme-immunoassay*

A typical sensitivity curve for DREK-EIA is shown in fig.2. Precision profile studies [18] indicated a coefficient of variation inferior to 10% in the interval between 2 and 250 fmol/well. The minimum detectable value (considered to be the dermenkephalin quantity that decreases the B_0 value 3 standard deviations) is of less than 2 fmol/well, i.e. 2 pg dermenkephalin. The specificity of the selected anti-DREK antiserum was investigated using various synthetic dermenkephalin analogs (table 1). The low cross reactivity (CR) values (<0.1%) of analogs where D-Met in the second position was replaced with either L-Met or D-Ala indicate the strict need of D-Met for antibody recognition of DREK. Changes occurring at the carboxy end were much less critical as shown by the relatively high CR values (60–75%) when Asp-NH_2 in seventh position was replaced with either Asp-OH or Asn-NH_2, or when Met in the sixth position was either oxidized or replaced with D-Met. Dermorphin presented no significant cross reactivity (<0.01%). The minimum structure to maintain high antibody recognition (20%) is DREK-(1-6)-NH_2, stepwise deletion of amino acids at dermenkephalin's carboxy end led to proportional loss of cross

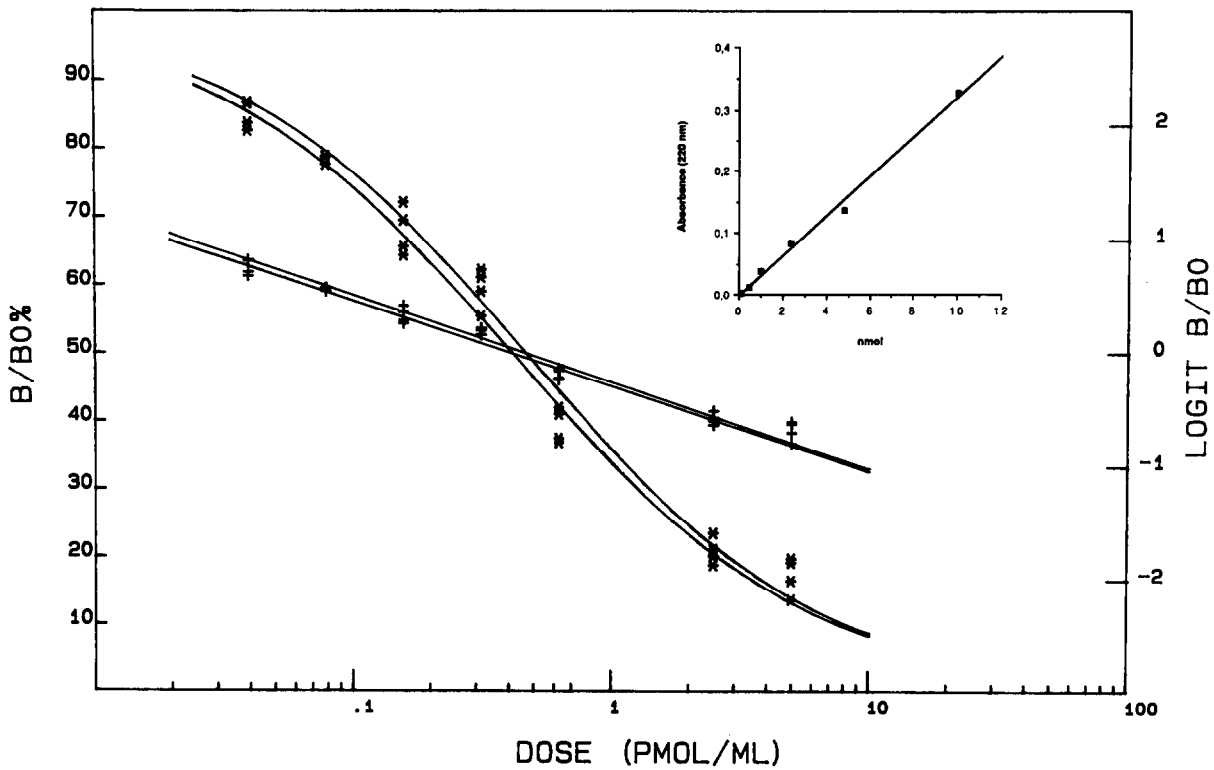

Fig.2. Typical standard curve obtained for routine EIA. B and B_0 represent the bound enzyme activity measured respectively in the presence or absence of synthetic DREK and LOGIT B/B_0 is the corresponding linear transformation. The parallel intermingled curve was obtained with the purified natural counterpart. (Inset) Synthetic dermenkephalin was used to construct a calibration curve where optical density at 220 nm was plotted against nmol DREK.

Table 1

Cross reactivity of dermenkephalin analogs

No.	Peptide sequence	Abbreviated	%CR[a]
P1	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	DREK	100
P2	Tyr-L-Met-Phe-His-Leu-Met-Asp-NH_2	[L-Met^2]-DREK	0.05
P3	Tyr-D-Ala-Phe-His-Leu-Met-Asp-NH_2	[D-Ala^2]-DREK	0.02
P4	Tyr-D-Met(o)-Phe-His-Leu-Met(o)-Asp-NH_2	[D-Met^2(o),Met^6(o)]-DREK	30
P5	Tyr-D-Met-Phe-His-Leu-Met-Asp-OH	DREK-(OH)	60
P6	Tyr-D-Met-Phe-His-Leu-Met-Asn-NH_2	[Asn^7]-DREK	60
P7	Tyr-D-Met-Phe-His-Leu-Met(o)-Asp-NH_2	[Met^6(O)]-DREK	75
P8	Tyr-D-Met-Phe-His-Leu-D-Met-Asp-NH_2	[D-Met^6]-DREK	60
P9	Tyr-D-Met-Phe-His-Leu-Met-NH_2	DREK-(1-6)-NH_2	20
P10	Tyr-D-Met-Phe-His-Leu-NH_2	DREK-(1-5)-NH_2	2
P11	Tyr-D-Met-Phe-His-NH_2	DREK-(1-4)-NH_2	0.05
P12	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	DRM	<0.01

[a] Using the Abraham criterion [22], the cross reactivity (CR), expressed in terms of percent cross reactivity (%CR), is defined as the percent molar concentration at 50% B/B_0 of the peptide tested compared to that of DREK whose %CR is set to be 100

reactivity and oxidized Met at both second and sixth positions retained 30% cross reactivity.

2.5. *Preparation of biological sample*

Five dry skins (1 g/skin) of the frog *Phylomedusa sauvagei* were minced with scissors and extracted twice for a week with 20 vols (v/w) of 80% methanol/water at 4°C under continuous stirring. Combined extracts were centrifuged for 40 min at 40000 × *g* and the supernatant evaporated under vacuum.

2.6. *Chromatographic characterization of immunoreactive material*

The dried methanol extract was dissolved in 10% acetic acid and fractionated on a previously calibrated gel filtration Sephadex G15 column. Immunoreactive fractions that coeluted with synthetic DREK were pooled, freeze dried, dissolved in 0.1% trifluoroacetic acid/water and refractionated on a RP-HPLC (Lichrosorb C18, 10 μm, 250 × 4.6 mm) column. Elution was achieved with a 0–60% linear gradient of acetonitrile in 0.1% TFA/water along 60 min at a flow rate of 1 ml/min (fig.3A). Immunoreactive fractions that coeluted with synthetic DREK were pooled, freeze dried, dissolved in 25% acetonitrile/water containing 0.1% TFA and refractionated on the same column but eluted with a 25–35% linear gradient of acetonitrile in 0.1% TFA/water along 50 min (fig.3B). Immunoreactive fractions that coeluted with synthetic DREK were submitted to a final purification step on a Lichrosorb C18, 5 μm (250 × 4.6 mm) column eluted with a slow linear gradient of 25–32% acetonitrile in 0.1% TFA/water for 60 min (fig.3C).

2.7. *Sequence analysis*

Sequence determination was carried out on an Applied Biosystems 470 gas-phase peptide sequenator. Phenylthiohydantoin amino acids were detected with an on-line Applied Biosystems 120 A analyzer. Data collection and analysis were performed with an Applied Biosystems 900 A module calibrated with 25 pmol phenylthiohydantoin amino acids standards.

2.8. *Opioid-binding assay*

The preparation of the particulate rat brain membrane fraction has been previously described [8]. Binding assays were performed at 24°C in 50 mM Tris-HCl, pH 7.4, plus 0.1% BSA and 0.01% bacitracin as described [9,11] using [^{3}H]DTLET (60 Ci/mmol, CEN Saclay, France) or [^{3}H]dermorphin (52 Ci/mmol) [8] as primary ligands. All determinations were performed in duplicate. The 50% inhibitory concentration values (IC_{50}) were obtained from non-linear least-squares regression of a 2 parameters logistic equation of the % specific binding versus logarithm of the dose curves. Inhibitory constants K_i were calculated from the Cheng-Prusof equation [19].

3. RESULTS AND DISCUSSION

Using a series of RP-HPLC runs operating with successively slow gradients of the same solvent system, the skin extract was gradually fractionated and the purification scheme is summarized in fig.3.

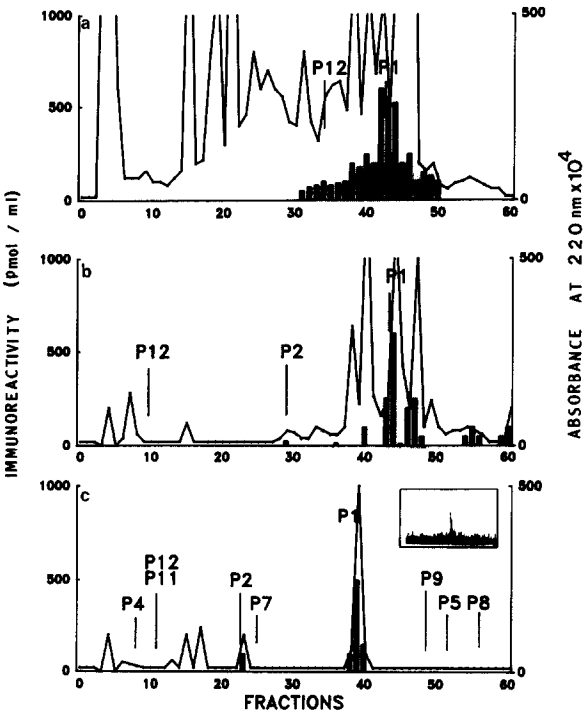

Fig.3. Purification scheme of DREK from skin extract of *Phyllomedusa sauvagei* and the elution positions of some synthetic analogs (refer to table 1). Absorbance is represented as straight lines and the immunoreactive fractions are represented as bars. (a) Immunoreactive fractions that co-eluted on G15 with synthetic DREK were recovered and injected onto a Lichrosorb C18, 10 μm (250 × 4.6 mm) column and eluted with a linear gradient of 0–60% acetonitrile along 60 min at a flow rate of 1 ml/min. (b) HPLC of fractions 41–44 from chromatogram A eluted with a linear gradient of 25–35% acetonitrile. (c) HPLC of fractions 43–44 from chromatogram B injected onto a Lichrosorb C18, 5 μm (250 × 4.6 mm) column and eluted with a linear gradient of 25–32% acetonitrile. (Inset) Mass spectrum of P1 (0.25 μg natural DREK by EIA) showing a peak corresponding to [M+H] = 956.

Synthetic dermenkephalin, dermorphin and related analogs were used as molecular markers. Two identification criteria were used to search for natural dermenkephalin. Fractions collected at each HPLC run were tested with the DREK-EIA procedure and the immunoreactive fractions that co-eluted with synthetic dermenkephalin were selected to undergo further fractionation. The other immunoreactive fractions observed (fig.3) due to cross reactivity of DREK-antibodies with DREK-related peptides were separated in the subsequent runs and their characterization will be

reported elsewhere. The immunoreactive fractions that co-eluted with synthetic DREK after the third purification step (fig.3C) were used for chemical and pharmacological analysis. On the basis of optical density and EIA (fig.2), we estimated the extract to yield 2.4 μg DREK (at least 80% pure)/g dry skin.

The sequence of P1 (fig.3c), the endogenous immunoreactive peptide (0.5 μg by EIA) was determined up to the seventh residue as Tyr-Met-Phe-His-Leu-Met-Asp by automated Edman degradation with a gas-phase sequencer. Since the sequence analysis does not yield information on the presence of a C-terminal amide group, the endogenous heptapeptide (0.25 μg by EIA) was further subjected to fast atom bombardment mass spectrometry. An unequivocal pseudo-molecular ion $[M+H]^+ = 956$ was observed (inset fig.3c) in the positive ion mode corresponding precisely to that expected theoretically for the sequence Tyr-Met-Phe-His-Leu-Met-Asp-NH_2. Moreover, synthetic DREK-OH (P2) behaved chromatographically differently from natural and synthetic dermenkephalin (fig.3c).

The presence of a D-methionine at the second position was unambiguously established by pharmacological, immunological and chromatographical criteria. The endogenous DREK isolated from skin extracts was tested for its ability to displace the prototypical δ-opioid ligand [^{3}H]DTLET [20] from rat brain membrane preparations. As shown in fig.4, natural DREK inhibited the high-affinity specific [^{3}H]DTLET binding to δ-opioid sites in a concentration dependent manner with a K_i of 1.06 nM and a quasi-Hill coefficient close to unity (table 2). The K_i of natural DREK was almost identical to that determined for synthetic DREK under similar experimental conditions (fig.4 and table 2). In contrast, the highly μ-selective opioid peptide [^{3}H]dermorphin [9] was displaced with very low potency by DREK (table 2). On the other hand, [L-Met^2]DREK was found to be virtually inactive in displacing both μ- and δ-opioid probes (table 2), a situation already encountered with [L-Ala^2]dermorphin [7]. As shown in fig.3, synthetic [L-Met^2]DREK (P2) behaves chromatographically very differently from DREK (P1) on RP-HPLC run under 3 distinct elution conditions. In addition, whereas natural DREK diluted parallel to synthetic DREK in DREK-EIA assay (fig.2), the

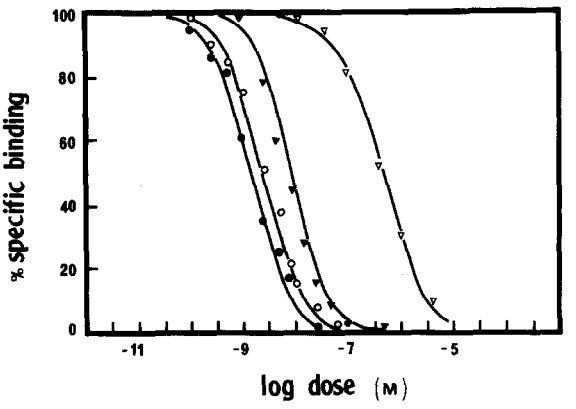

Fig.4. Inhibition of [^{3}H]DTLET specific binding (1 nM) to rat brain membranes (0.74 mg/ml of membrane proteins) by increasing concentrations of natural dermenkephalin (●), synthetic dermenkephalin (○), DTLET (▲) and dermorphin (△). The percentage of specific binding was calculated as $100 \times (B_s - B_n)/(B_o - B_n)$, where B_s and B_o are respectively the amount bound in the presence or absence of competing compound, and B_n is the non-specific binding, i.e., the binding in the presence of 1000 nM unlabeled DTLET. The solid lines are theoretical fits to a simple binding isotherm determined by non-linear least-squares regression analysis. The data shown are for a single representative experiment. Values for IC_{50} were determined by regression analysis based upon 2–4 independent experiments carried out in duplicate.

Table 2

Potencies of natural and synthetic dermenkephalin in inhibiting the binding at the δ ([^{3}H]DTLET, 1 nM) and the μ sites ([^{3}H]dermorphin, 0.5 nM) in homogenates of rat brain at 24°C in 50 mM Tris-HCl buffer, pH 7.4

Ligand	K_i[a] (nM)		$K_i(\mu)/K_i(\delta)$[b]
	[^{3}H]DTLET	[^{3}H]Dermorphin	
DTLET	6.0 (0.7)	25.5 (3.5)	4.2
Dermorphin	359 (31)	0.73[c] (0.02)	0.002
DREK (synthetic)	1.77 (0.08)	619[c] (56)	350
DREK (natural)	1.06 (0.11)	n.d.[d]	
[L-Met^2]DREK[e]	3141 (449)	3374 (151)	1.1

[a] The inhibitory constant K_i was calculated from IC_{50} values using the Cheng-Prussof equation [19]. Reported values are the mean of 2–4 experiments carried out in duplicate. Values in parentheses are SEM

[b] The selectivity ratio at the δ site is expressed as the ratio, K_i for μ (vs [^{3}H]dermorphin)/K_i for δ (vs [^{3}H]DTLET)

[c] Taken from [11]

[d] Not determined due to low amount of natural dermenkephalin

[e] Taken from [21]

very low cross reactivity value (0.05%) of [L-Met^2]DREK in DREK-EIA indicated the strict requirement of the D-configuration of the methionine residue in position 2 for antibody recognition (table 1).

In conclusion, we have isolated from amphibian skin an heptapeptide having a D-Met residue in position 2 which stereospecifically binds with high affinity and specificity to the δ-opioid receptor. The identity of this new pro-dermorphin related peptide was established by co-chromatography with synthetic DREK on reverse-phase HPLC, immunological analysis, sequence determination, mass spectrometry and by pharmacological assay. Pro-dermorphin thus offers a surprising example of an opioid biosynthetic precursor that simultaneously generates two D-amino acid containing peptides which behave as highly potent and specific agonists for the μ (morphine)- and δ (enkephalin)-opioid receptors, respectively. In addition, because DRM and DREK have no structural features in common with the sequence of all the hitherto known opioid peptides, they should be useful tools for identifying conformational determinants for high affinity and selective binding of opioids to the μ- and δ-receptors [21].

NOTE ADDED IN PROOF

During the preparation of this manuscript, Kreil et al. [23] have reported on the isolation of a peptide from amphibian skin whose structure and pharmacological activity appear to be essentially similar to those of dermenkephalin. This peptide was named deltorphin by these authors.

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