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EVIDENCE FOR PRO-DERMORPHIN PROCESSING PRODUCTS IN RAT TISSUES

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Summary. Dermorphin (Tyr-D.Ala-Phe-Gly-Tyr-Pro-Ser-NH2) and dermenkephalin (Tyr-D.Met-Phe-His-Leu-Met-Asp-NH2), two powerful opioid peptides issued from a common biosynthetic precursor,were recently isolated from the skin of the frog *phyllomedusa sauvagii*. Since many amphibian's skin secretory peptides or their homologues are found in the mammalian central nervous system and gastrointestinal tract, dermorphin and dermenkephalin may have counterparts in mammals. We have prepared antibodies directed against dermorphin, dermenkephalin and the spacer sequence lying between them in the progenitor form and developed sensitive enzyme immunoassays that detect under the picogram level to verify the occurrence of these peptides in rat tissues. Immunocytochemical studies of rat brain sections revealed a similar distribution of immunoreactivities both at the regional and the cellular levels when either one of these three anti-sera was used. Characterization of the immunoreactive peptides was achieved with molecular sieve filtration followed with Reverse Phase High Performance Liquid Chromatography of various rat tissues extracts. Identification was achieved by immunological analysis and chromatographic comparison with synthetic peptides. Immunoreactive materials corresponding to dermorphin, dermenkephalin and the spacer peptide were detected in either brain, stomach or intestine, indicating processing of the dermorphin precursor in these tissues. Immunoreactive species of higher Mr were also detected in all three tissues and may represent extended forms or homologous peptides.

Amphibian peptides represent a useful tool in the identification of mammalian counterparts since frog skin may contain up to 100,000 times higher doses of biologically active peptides which may be present in the brain and gastointestinal tract of mammals in only minute quantities (ex: TRH and Tachykinines (1)). A cDNA library was recently prepared from the skin of the south american frog *Phyllomedusa sauvagii* and the primary structure of at least two dermorphin biosynthetic precursors was elucidated (2). One precursor (Fig. 1) is shown to code for a repetitive pattern of five extensively homologous sequences of about 35 aminoacids, one of which codes for dermenkephalin (DREK) and the other four code for dermorphin (DRM). The

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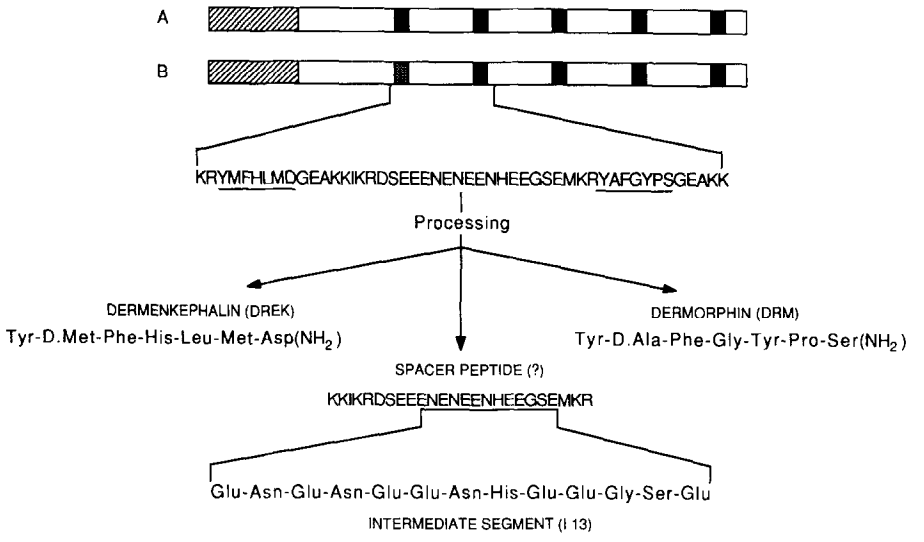

Figure 1: Schematic representation of the structural organisation of the dermorphin biosynthetic precursors in the frog *Phyllomedusa sauvagii* (2). The N-terminal dashed sequence is the predicted signal peptide sequence. One precursor (A) contains five DRM sequences while the second (B) contains one DREK (shaded) and four DRM (blackened) sequences. The aminoacid sequence (in one letter code) of a portion of the precursor B is shown to illustrate the peptides against which antibodies were directed.

other precursor presents five sequences coding for dermorphin only. Both peptides, dermorphin and dermenkephalin were isolated from the methanol extract of the *Phyllomedusa sauvagii* dry skin and their respective structures as well as their pharmacological profiles are now established (3-17). Unlike the mammalian opioid peptides known, i.e. enkephalins, dynorphins and endorphins, dermorphin and dermenkephalin do not contain the met- or leu-enkephalin motif in their sequences. Aside from being two powerful and the most selective known endogenous agonists respectively to the μ(morphine) and ∂(enkephalin) opioid receptors, they present the intriguing property of containing D-aminoacids incorporated at the second position of their sequences, even though they are coded for by usual L-aminoacid codons. The biosynthetic mode of incorporation of a D-aminoacid within a peptide sequence is unknown and may be of utmost importance should it occur in mammals as well. Preliminary results have been obtained showing the presence of dermorphin-immunoreactivity or bioactivity in rat and guinea pig tissues (19-21). However, the putative dermorphin-like species were never characterized, hence, the occurence of dermorphin or closely homologous sequences in mammalian tissues remains to be unambigously established.

Assuming the same processing events as for amphibians, processing of the dermorphin precursor(s) in mammals is expected to generate two amidated heptapeptides, DRM and DREK as well as whole or fragmented spacer peptides (Fig.1). We have prepared antibodies against dermorphin, dermenkephalin and against a 13 aminoacid sequence of the spacer peptide (I13), and used immunocytochemical and biochemical methods to detect and characterize these peptides in rat tissues. Results are discussed herein.

Materials and methods

Peptide synthesis: All peptides used were prepared in our laboratory as previously described (18) by stepwise manual solid-phase synthesis using the preformed symmetric anhydride technique. Homogeneity of synthetic products was assessed with analytical RP-HPLC, thin-layer chromatography, amino acid analysis and fast atomic bombardment mass spectrometry.

Antibodies preparation: The preparation of anti-dermorphin and anti-dermenkephalin antibodies and their respective enzymatic tracers has been previously described (12,13). I13 was coupled through its amino end to bovine serum albumine (BSA) for immunogen preparation.10µl glutaradehyde 25% were added to synthetic I13 (18mg) in 1ml 0,1M phosphate buffer pH 7 contaning 26mg BSA and left to react overnight in dark at 22°C under stirring, then dialysed against 0,9% NaCl. For the preparation of I13 enzymatic tracer, 50µl (1µmol) Difluorodinitrobenzene (DFDB) were added to react in dark at 22°C for two hours with 100µl containing 10 nmol synthetic I13 in 0,1M phosphate buffer pH 7 then dried under N2 stream, dissolved in 100µl water, extracted three times with ether to remove excess reagent and added to 500µl 0,1M borate buffer pH 9 containing 100µg G4 form of acetylcholinesterase. The mixture reacted overnight at room temprature then purified by molecular sieve chromatography.
Immunization and sera screening for antibodies were performed strictly as previously described (12,13) where the procedure as well as the characteristics of their respective enzyme immunoassays (EIA) were detailled. The specificity profile of antibodies as tested with EIA technique is summerized in table 1. The working dilutions of antisera and their respective sensitivities (B/B_o at 50%) were 1:20,000 and 10 fmol/well for DRM and DREK, 1:50,000 and 1000 fmol/well for I13.

Immunocytochemistry: Under deep chloral hydrate anesthesia (350 mg/kg), Wistar rats (200-300 gr) were perfused via the ascending aorta with 100ml of a washing solution containing 0,9% NaCl supplemented with 0,1% sodium nitrite as vasodilatator, followed with 800ml of 0,4% paraformaldehyde in 0,1M Sörensen buffer pH 7,4. Brains were dissected out and postfixed in the same fixative medium during two hours. After extensive washing in 0,15M Sörensen buffer, 50µm sections were made with a vibratome and processed for the indirect avidin-biotin peroxidase method. Free floating sections were preincubated in 1% normal goat serum diluted in Sörensen buffer containing 0,1% Triton-X-100. Sections were then incubated overnight at 4°C with an antiserum at dilutions of 1:1000 for either DRM or DREK antisera and 1:3000 for I13 antiserum. After extensive washing with buffer, sections were incubated with anti-rabbit IgG coupled to horseradish peroxidase (HRP) complex (Vectastain laboratories). HRP activity was then revealed with the 3,3' diaminobenzidin method. Immunocytochemical controls were made with 2 hours preincubation of the antibodies, at concentrations used for immunocytochemistry, with 1µM of the corresponding immunogen, at room temprature, before being added to sections and treated as described above.

Preparation of biological sample and chromatographic characterization of immunoreactive material: Brain (15g), stomach (12g) and about 10cm intestine including duodenum (22g) of ten 180g Sprag Dawley male rats were dissected after decapitation and ground in liquid N2. The ground tissues were then homogenized in 10 volumes 10% acetic acid, centrifuged for 30min. at 35,000 rpm and supernatant lyophilized. The dried extract was dissolved in 10% acetic acid and fractionated on a previously calibrated gel exclusion (Sephadex G-50 superfine) column. Fractions (3ml) were evaporated under vacuum, dissolved in 500µl 0,1% trifluoracetic acid (TFA)/water from which 50µl aliquot were assayed. Immunoreactive fractions (47-60) that coeluted with the Vt marker (Ferricyanure) were pooled, evaporated under vacuum, dissolved in 0,1% (TFA)/water and refractionated on an analytical RP-HPLC column (Ultrasphere 5µm spherical 80A° pore C18 4,6x250mm from Beckman). Elution was achieved with an initial 5min. wash in 0,1% TFA/water then with a 0-60% linear gradient of acetonitrile containing 0,07% TFA at a flow rate of 1ml/min. 1ml fractions were collected, lyophilized and dissolved in 100µl EIA buffer for assay.

Results and Discussion

Immunocytochemical detection of pro-dermorphin processing products in rat brain. When either one of the three antibodies was used in rat brain sections, a similar pattern of immunostaining was observed as shown for the area postrema in Figure 2. Depending upon the relative affinity and titer of the antibodies, the intensity of the reaction was not equivalent, however, the regional and cellular distribution of the reaction was similar and essentially localized around capillaries, in numerous fibers and in ependymal cells of the fourth ventricle. A detailed immunocytochemical study showing the wide distribution of these peptides in the whole brain, including regions involved in pain control, will be reported elswhere (Lopez et al, submitted). Control experiments have shown a dose-dependent decrease of the immunoreactivity when antibodies were preincubated with the corresponding immunogen two hours before addition to sections (Fig. 2D). Moreover, the demonstration of a co-localization of DRM, DREK and I13 is indeed a fine control itself and the use three antibodies directed against different peptidergic sequences issued from a common precursor strongly diminishes the possibility of non-specific cross reaction.

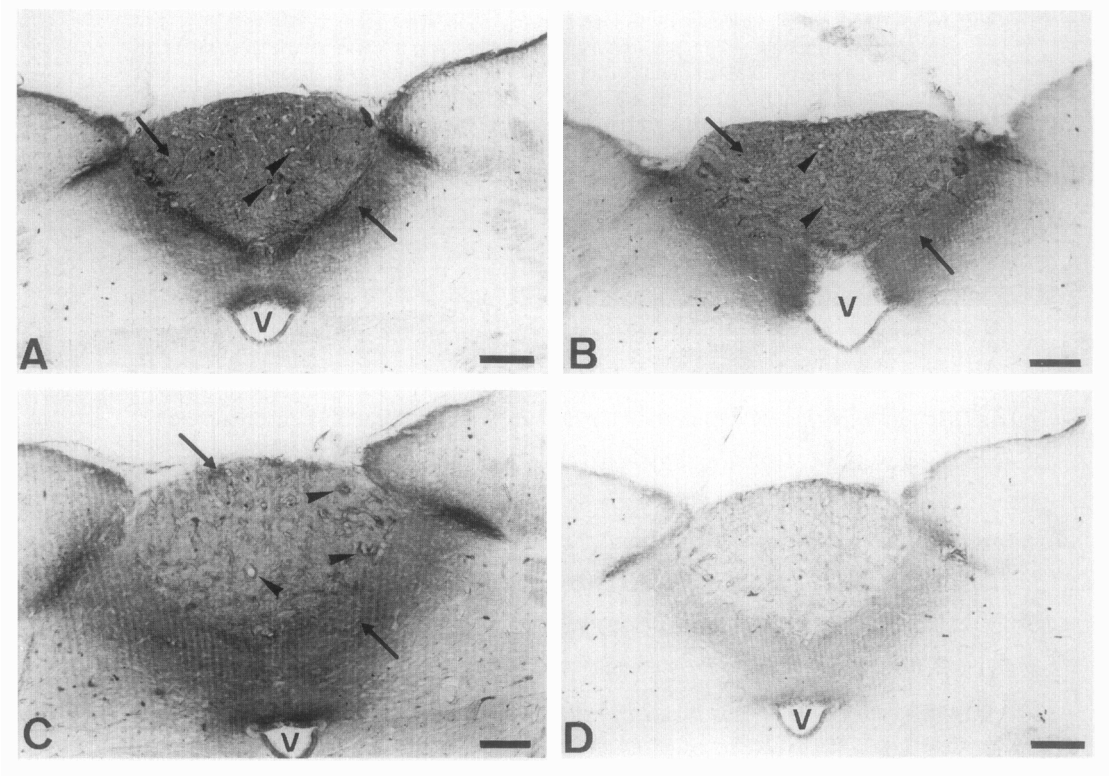

Figure 2: Immunoreactivities in the area postrema and adjacent regions of the rat brain, using either anti-dermenkephalin (A), anti-I13 (B) or anti-dermorphin (C) antisera. Preabsorption of antisera with 1μM of the corresponding immunogen led to a marked decrease of the immunoreactivity (exemple of DRM in D). Scale bars = 500μm. The similar pattern of immunoreactivities in A, B, and C is to be noticed. Arrows and arrow heads point to immunoreactive fibres and capillaries, respectively. Periependymal cells around the fourth ventricle (V) are also labelled.

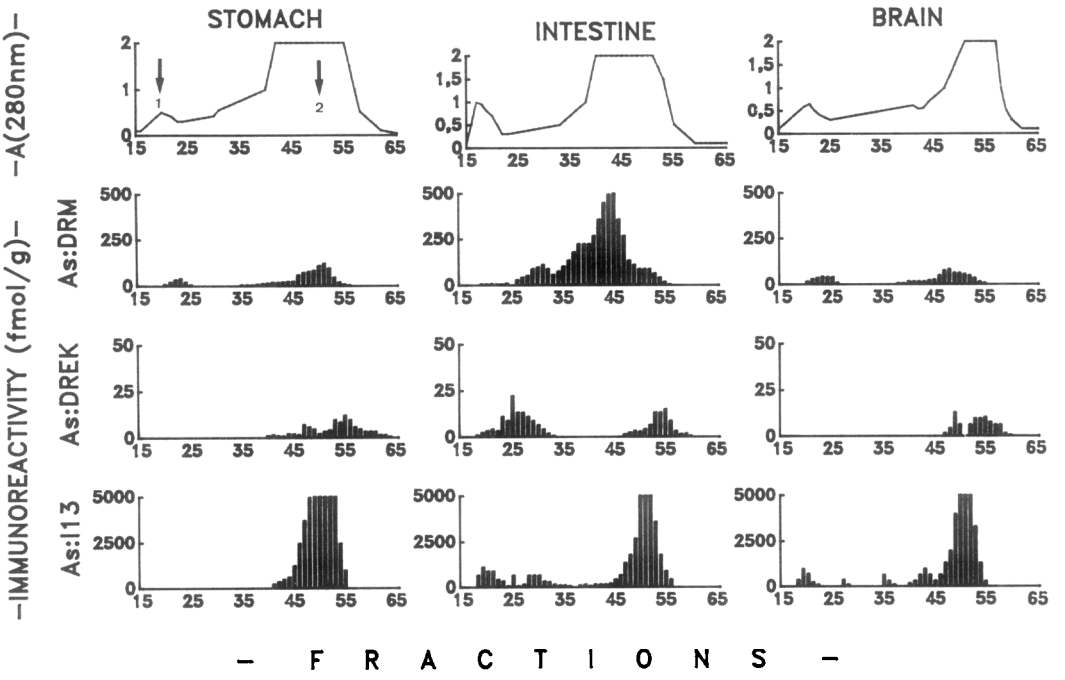

Figure 3: Fractionation profiles of rat tissues extracts resolved by gel exclusion chromatography (Sephadex G-50 superfine, 1,5 x110 cm) using a 10% acetic acid solvent system at a flow rate of 10ml/hour and collected 3ml/fraction. Continuous absorbance (top line) was monitored at 280nm and immunoreactivity was measured by EIA on aliquots of each fraction using dermorphin, dermenkephalin and I13 antisera (As:DRM, As:DREK and As:I13, respectively). Values are expressed in fmol immunoreactivity per gram fresh tissue. The void and total volumes were determined by elution of BSA (arrow 1) and Ferricyanure (arrow 2). Synthetic dermorphin and dermenkephalin eluted within the total volume.

Characterization of the DRM immunoreactivity. The fractionation of rat tissues extracts on a gel exclusion G-50 column, when tested with the dermorphin antiserum, revealed immunoractive material at the total volume (Vt) for either brain, stomach or intestine (Fig. 3, line As:DRM) as expected for a peptide of the dermorphin size (Mr=803). Validation was achieved by coelution of synthetic dermorphin under the same experimental conditions. Further support for structural identity between synthetic and endogenous DRM was obtained with HPLC analysis. Immunoreactive fractions that coeluted with synthetic DRM on the G-50 column (fractions 47-60) were pooled and injected onto an analytical C18 RP HPLC column. The immunoreactivity was recovered as a unique sharp peak (Fig.4) eluting, again, at the position of synthetic DRM. Note that these experimental conditions resolved DRM from DRM analogs as distinct peaks (legend Fig.4). The antigenic similarity of synthetic DRM with the endogenous peptide was further assessed by competition experiments in the enzyme immunoassay where both forms diluted parallel to synthetic standard EIA curve (not shown). These results were obtained with either brain, stomach or intestine. Other immunoreactive materials were detected on the G-50 column eluting either at the void volume or before the total volume. When processed on HPLC, these higher Mr immunoreactive forms did not coelute with DRM or with shorter synthetic DRM analogs nor did they dilute parallel to synthetic DRM-EIA curve.

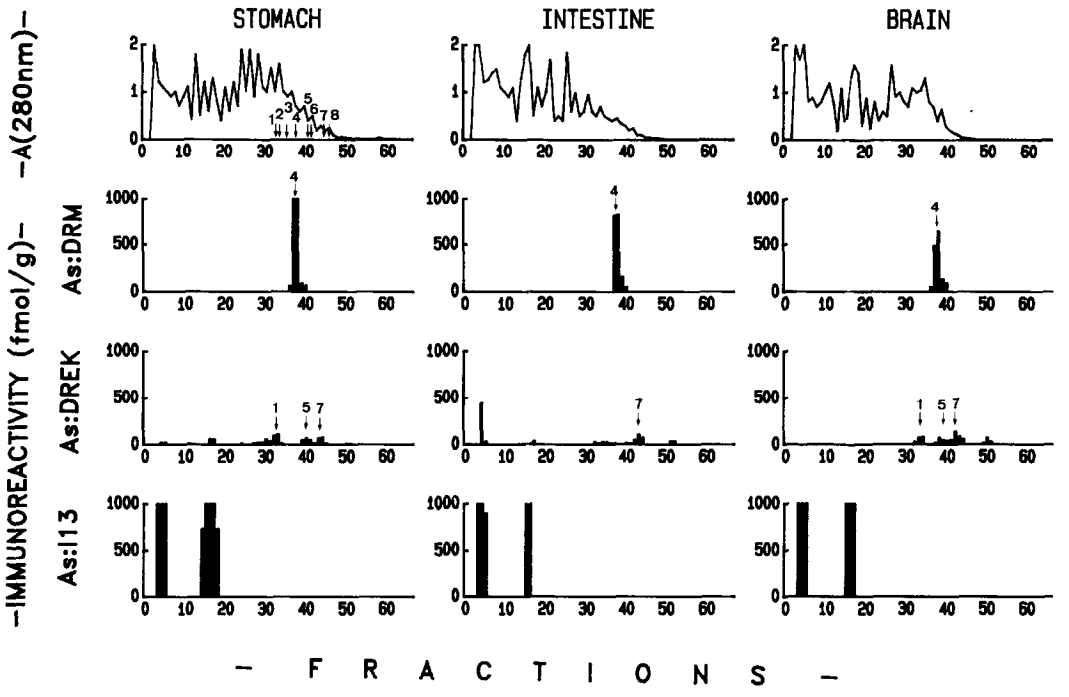

Figure 4: Reverse phase HPLC profiles of immunoreactive fractions that eluted within the total volume of the G-50 column. Fractions 47-60 (chromatograms Fig. 3) of either intestine, stomach or brain were pooled, concentrated and injected onto an analytical (Ultrasphere 5µm, C18, 4,6 x250 mm) column. Elution was with an initial 5min. wash of 0,1% TFA/water followed with a 0-60% linear gradient of acetonitrile in 0,1% TFA/water for 60min. 1ml fraction were collected at a flow rate of 1ml/min. Continuous absorbance (top line) was monitored at 280nm and immunoreactivity was measured by EIA on aliquots of each fraction using dermorphin, dermenkephalin and I13 antisera (As:DRM, As:DREK and As:I13, respectively). Values are expressed in fmol immunoreactivity per gram fresh tissue. Scales were normalized to allow for comparison. Elution position of some DRM and DREK synthetic analogs are indicated with arrows: 1) 33,2min. for [Met^2(o),Met^6(o)]-DREK; 2) 33,5min. for [L-Ala^2]-DRM; 3) 36,4min. for DRM acid; 4) 37,5min. for DRM; 5) 40,2min. for [Met^6(o)]-DREK; 6) 40,5min. for [L-Met^2]-DREK; 7) 43,5min. for DREK; 8) 44,5min. for DREK acid.

Characterization of the DREK immunoreactivity. When tested with the dermenkephalin antiserum, the G-50 columns revealed immunoreactive material coeluting with synthetic DREK (Mr=955) slightly past the total volume (Fig. 3, line As:DREK). In attempting to further characterize this material on RP HPLC, innitial immunoreactivity was recovered in several peaks (Fig. 4). Three of these were found to elute at the elution positions of either synthetic DREK (43,5min.), oxydized DREK at one methionine (40,2min.) and oxydized DREK at both methionines (33,2min.). The same situation was encountered in *Phyllomedusa sauvagii* skin extract from which dermenkephalin was isolated (13). The alleged DREK peak dilluted parallel to synthetic DREK standard EIA curve but not the oxydized forms. These oxydized forms cross-react strongly with the DREK antiserum (table 1) and conserve, though to a lesser extent, their receptor binding capacity (15); however, whether they occur naturally or they are artefactually generated during extraction, is unknown.

Table 1- Cross reactivity * (CR) of analogs and opioid peptides

	Compounds**	As:DRM	As:DREK	As:I13
1.	Y-A-F-G-Y-P-S-NH2	100	<0.01	<0.01
2.	Y-A-F-G-Y-P-S-NH2	0.1	<0.01	<0.01
3.	A-F-G-Y-P-S-NH2	< 0.01	<0.01	<0.01
4.	Y-A-F-G-Y-P-S	7	<0.01	<0.01
5.	Y-A-F-G-Y-P-S	1000	<0.01	<0.01
6.	Y-A-F-G-Y-P-NH2	17	<0.01	<0.01
7.	Y-A-F-G-Y-NH2	0.4	<0.01	<0.01
8.	Y-A-F-G-NH2	0.07	<0.01	<0.01
9.	Y-A-F-NH2	0.01	<0.01	<0.01
10.	Y-A-F-G-Y-P-S-C-NH2	10	<0.01	<0.01
11.	Y-A-F-G-Y-P-S-G-K-R	6	N.D.	N.D.
12.	Y-A-F-G-Y-P-S-G-E-A-K-K	7	N.D.	N.D.
13.	Y-M-F-H-L-M-D-NH2	<0.01	100	<0.01
14.	Y-M-F-H-L-M-D-NH2	<0.01	0.05	<0.01
15.	Y-M-F-H-L-M-D	<0.01	60	<0.01
16.	Y-A-F-H-L-M-D-NH2	N.D.	0,02	<0.01
17.	Y-M(o)-P-H-L-M(o)-D-NH2	<0.01	30	<0.01
18.	Y-M-F-H-L-M-N-NH2	<0.01	60	<0.01
19.	Y-M-F-H-L-M(o)-D-NH2	<0.01	75	<0.01
20.	Y-M-F-H-L-M-D-NH2	<0.01	60	<0.01
21.	Y-M-F-H-L-M-NH2	<0.01	20	<0.01
22.	Y-M-F-H-L-NH2	N.D.	2	<0.01
23.	Y-M-F-H-NH2	N.D.	0.05	<0.01
24.	E-N-E-N-E-E-N-H-E-E-G-S-E	<0.01	N.D.	100
25.	Met-Enkephalin	< 0.01	N.D.	N.D.
26.	[D-Ala^2, D-Leu^5]enkephalin	< 0.01	N.D.	N.D.
27.	ß-Endorphin	< 0.01	N.D.	N.D.
28.	Dynorphin [1-17]	< 0.01	N.D.	N.D.

* Using the Abraham criterion (22), the cross reactivity is expressed in terms of percent Cross Reactivity (%CR) defined as the percent molar concentration at 50% B/Bo of the peptide tested compared to that of DRM, DREK or I13 respectively, whose %CR is set to be 100. ** Amino Acids are represented in the one letter code; D-Amino Acids are underlined. N.D.=not determined.

Characterization of the I13 immunoreactivity. As for DRM and DREK, the total volume of the G50 columns of all three tissues contained I13 immunoreactivity (Fig. 3, line As:I13). When applied to RP HPLC, the initial immunoreactivity was resolved into two hydrophilic peaks (Fig. 4, line As:I13). Neither peak diluted parallel to the synthetic I13 standard EIA curve, indicating some antigenic unsimilarities. This differential antigenic recognition may be explained from inspection of the progenitor aminoacid composition (Fig.1), although the size of the intermediate peptides cannot be precisely predicted, being more susceptible to degradation, one may expect their processing to yield a variety of small hydrophilic peptides wich may cross react with As:I13 but would hardly dillute parallel or be retained on HPLC under these experimental conditions.

Conclusions

We have previousely reported on the use of DRM and DREK antisera. Being specific (as shown by the cross reactivity profiles in table 1) and sensitive (detecting under the picogram level), they

were successfully used for the isolation of both dermorphin and dermenkephalin from *Phyllomedusa sauvagii* skin extract. The results presented herein, together with previous reports (12,19-21) bring strong arguments in favor of an endogenous synthesis and processing of pro-dermorphin in mammalian tissues. Rat tissues examined contained peptides whose chromatographic properties and immunological characteristics were indistinguishable from those of authentic dermorphin and dermenkephalin. Since dermorphin and dermenkephalin are produced via proteolytic processing of larger biosynthetic precursors (2), the occurrence of peptides which could result from partial processing or yet unprocessed prohormone is to be expected. In that regard, immunoreactive species eluting ahead of the total volume or within the void volume of the G50 column may represent C-and/or N-terminally extended forms of dermorphin or intact precursors, respectively. The nature of these materials is curently under investigation and preliminary results support this hypothesis as cell bodies and distal part of neurons revealed to be immunoreactives with antibodies directed against elongated forms of dermorphin. Finally, intestine extract presents a more complex immunoreactive profile as compared to brain and stomach extracts (Fig. 3), suggesting a differential processing pattern in this tissue.

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