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Dermenkephalin (Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2): A Potent and Fully Specific Agonist for the δ Opioid Receptor

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SUMMARY

Dermorphin, Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 is an extraordinarily potent and highly μ-selective opioid heptapeptide isolated from amphibian skin. It is unique among peptides synthesized by animal cells in having an amino acid residue in the D-configuration. At least two different preprodermorphin cDNAs were cloned from skin of *Phylomedusa sauvagei*; their predicted amino acid sequences contained four to five homologous repeats of 35 amino acids, each repeat including one copy of the dermorphin progenitor sequence. Tyr-Ala-Phe-Gly-Tyr-Pro-Ser-Gly, flanked by Lys-Arg at the amino end and by Glu-Ala-Lys-Lys at the carboxyl end [*Science (Wash. D. C.)* **238**:200–202 (1987)]. The D-Ala in position 2 in dermorphin is encoded by a usual Ala codon in the precursor sequence. Of the two prodermorphin molecules, one has a dermorphin copy replaced with a distinct heptapeptide sequence, Tyr-Met-Phe-His-Leu-Met-Asp, sandwiched by the same processing signals. Assuming the same pathway as for the release of dermorphin, processing of this precursor may yield, beside dermorphin, a copy of a new peptide, Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2. We have synthetized this peptide together with its (L-Met^2)-counterpart and evaluated their respective opioid receptor selectivity in the mouse vas deferens and guinea pig ileum assays and in rat brain membrane binding assays. Overall, the data collected demonstrate that the putative prodermorphin product Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 named dermenkephalin, behaves as a potent δ opioid agonist exhibiting high affinity and high selectivity for the δ opioid receptor. Prodermorphin, thus, offers a surprising example of an opioid biosynthetic precursor that might simultaneously generate highly potent and fully selective agonists for the μ- (morphine) and the δ (enkephalin) opioid receptors, respectively. In addition, because dermenkephalin has no structural features in common with the sequence of all the hitherto known opioid peptides, it should be a useful tool for identifying conformational determinants for high affinity and selective binding of opioids to the δ receptor.

The dermatous granular glands of amphibians synthesize an extraordinarily rich variety of biologically active peptides (1, 2). It is currently thought that most of these peptides have counterparts, either identical or homologous, in the mammalian brain and gastrointestinal tract. Among secretory peptides isolated from amphibian skin, the potent opioid heptapeptide dermorphin (3) is endowed with outstanding structural and biological features. Although the primary structure of dermorphin, Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 is basically different from that of the mammalian opioid peptides, its biological activity outclasses all hitherto known natural or synthetic opioids and opiates, being up to 1000 times more potent than morphine in producing long lasting analgesia upon intracerebroventricular administration in rodents (3–5). Dermorphin is unique among peptides synthesized by animal cells in having an alanine in position 2 that is in the D-configuration. (L-Ala^2)Dermorphin is virtually inactive both *in vivo* and *in vitro* (6, 7). Moreover, *in vitro* binding studies have shown dermorphin to be the first naturally occuring opioid peptide that exhibits high affinity and full selectivity for the μ (morphine) opioid receptor (8, 9).

Recent evidence has been obtained showing that dermorphin is synthesized via larger biosynthetic precursors (Fig. 1) (10), thus establishing this peptide as the first example of a D-amino acid-containing peptide to be synthesized by the ribosomal route. The predicted amino acid sequences of prodermorphins contained four or five homologous repeats of 35 amino acids, each of these including a copy of dermorphin flanked by the typical prohormone-processing signal, Lys-Arg at the amino end and Gly-Glu-Ala-Lys-Lys at the carboxyl end (Fig. 1) (10). Excision of the paired Lys residues at the C-terminal end, followed by cleavage of the extra dipeptide by a carboxypeptidase or a dipeptidyl carboxypeptidase, would expose the Gly residue required for carboxamidation of dermorphin (10). In the corresponding cloned cDNAs, a usual alanine codon occurred at the position where D-Ala is present in mature dermorphin. This observation suggests three possible ways for

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ABBREVIATIONS: DAGO, Tyr-D-Ala-Gly-MePhe-Gly-ol; DPDPE, (D-Pen^2,D-Pen^5)-enkephalin; DTLET, (D-Thr^2,Leu^5)-enkephalyl-Thr^6; EKC, ethylketocyclazocine; GPI guinea pig ileum; MVD, mouse vas deferens; HPLC, high performance liquid chromatography.

incorporation of D-Ala into dermorphin, (i) recognition of D-Ala by a tRNA (L-Ala) or by an as yet unknown tRNA, (ii) an L to D isomerization of L-Ala once it is bound to a regular tRNA (L-Ala), or (iii) a posttranlational L to D isomerization of Ala once it is incorporated into dermorphin. The L to D isomerization is, hence, an intriguing subject and surely an opportunity for challenging breakthroughs in modern biochemistry.

Interestingly, one of the dermorphin precursor sequences has its first dermorphin copy replaced with a distinct heptapeptide sequence, Tyr-Met-Phe-His-Leu-Met-Asp, sandwiched by the same processing signals (Fig. 1). Assuming the same processing pathway as for the release of dermorphin, processing of such a precursor is expected to yield, beside four copies of dermorphin, a new peptide, Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2, with unknown biological activities. We have, therefore, synthesized this predicted sequence together with its (L-Met^2)- counterpart by the solid phase method, in order to examine their opioid receptor binding profiles *in vitro* and their pharmacological activities both *in vitro* and *in vivo*. Results demonstrated that Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2, named dermenkephalin, acts as a highly potent and fully selective agonist for the δ opioid receptor, whereas (L-Met^2)dermenkephalin is virtually devoid of opioid activity.

Materials and Methods

Chemicals. [3,5-^{3}H]DAGO (47 Ci/mmol) was purchased from the Commissariat à l'Energie Atomique (Saclay, France). [3,5-^{3}H]DPDPE (51.3 Ci/mmol) and [^{3}H]EKC, (27 Ci/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA). Tritiated dermorphin (52 Ci/mmol) was prepared and purified as described (8). The radiochemical purity of tritiated ligands was checked by HPLC before use and found to be >98%. Unlabeled DAGO and DPDPE were from Peninsula Laboratories (Belmont, CA).

Peptide synthesis. Dermorphin, Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 (dermenkephalin), and (L-Met^2)dermenkephalin were prepared by stepwise manual solid phase synthesis on benzhydrylamine polymer, using the preformed symmetric anhydride technique as described (7, 9). After treatment with liquid HF and ether extraction, the peptides were purified by a combination of Sephadex gel filtration, ion exchange chromatography, and preparative HPLC, as reported (11). Homogeneity of synthetic peptides was assessed by analytical HPLC, thin layer chromatography, amino acid analysis, and fast atom bombardment mass spectrometry (11).

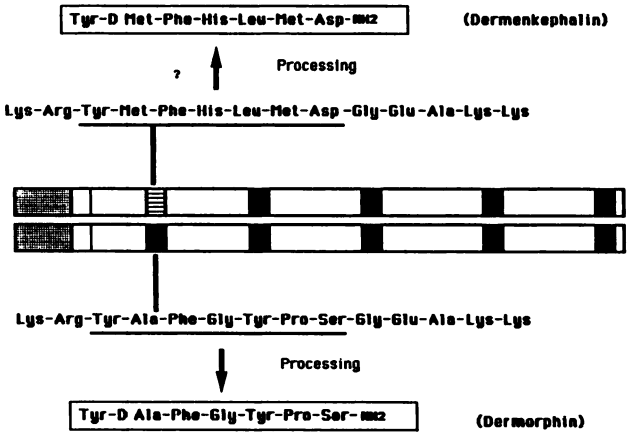

Fig. 1. Schematic representation of the structural organization of the dermorphin biosynthetic precursors from the frog *Phyllomedusa sauvagei* (10). The N-terminal *dashed sequence* is the predicted signal peptide sequence. Positions of dermorphin progenitor sequence, Tyr-Ala-Phe-Gly-Tyr-Pro-Ser, which 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 might yield dermenkephalin, i.e., Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2.

Opioid binding assays. The preparation of the particulate rat brain membrane fraction has been previously described (8, 12). Binding assays were performed at 24° in 50 mM Tris·HCl, pH 7.4, plus 0.1% bovine serum albumin and 0.01% bacitracin, as described (8). Each assay contained, in a final volume of 2 ml, the membrane preparation (1.38 mg of membrane proteins) and the tritiated ligand at the desired concentration, with or without unlabeled ligand. The nonspecific binding was determined in the presence of 1 μM concentrations of the unlabeled primary ligand. The tubes were incubated for the desired time period (1 hr for [^{3}H] DAGO, [^{3}H]dermorphin, and [^{3}H] EKC, 2 hr for [^{3}H] DPDPE) with stirring every 15 min. The binding reaction was terminated by rapid vacuum filtration through 0.1% polyethylenimine-coated Whatman glass fiber filters (GF/B). The filters were washed twice with 10 ml of cold 50 mM Tris·HCl, pH 7.4, 0.1% bovine serum albumin, and were transferred to vials containing 5 ml of PCS scintillation fluid (Amersham). Specific binding was considered to be the difference between the radioactivity trapped on the filters in the absence and that in the presence of 1000 nM unlabeled primary ligand. All determinations were performed in duplicate. The 50% inhibitory concentration values (IC_{50}) were obtained from nonlinear least squares regression to a two-parameter logistic equation of the per cent specific binding versus logarithm of the dose) curves. The inhibitory constants (K_i) of the various unlabeled ligands were calculated from the relation $K_i = IC_{50}/(1 + L/K_d)$ (13), where L is the concentration of the labeled ligand and K_d is its equilibrium dissociation constant, determined by saturation binding analysis. All binding experiments were performed with the same preparation of brain membranes and purified radioligands.

Pharmacological assays. The effect of intracerebroventricularlly administrated opiates or peptides on heat escape latency was assessed by the tail-flick method (14) using groups of 10 mice (male Swiss Webster, 20–25 g) per dose as described (15). Median antinociceptive dose (AD_{50}) and 95% confidence limits were calculated from the logarithm of the dose versus probit per cent analgesia) curves (15). Phar-

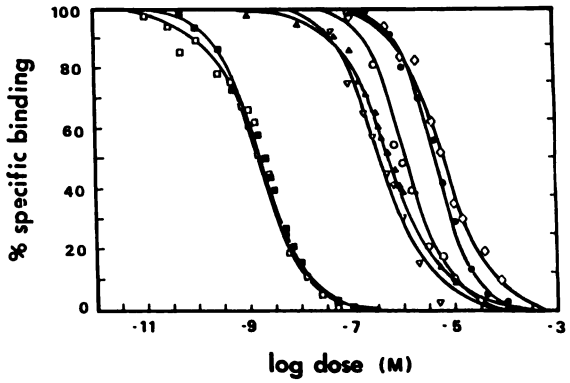

Fig. 2. Inhibition of [^{3}H]DAGO specific binding (0.5 nM) to rat brain membranes (0.74 mg/ml membrane proteins) by increasing concentrations of unlabeled DAGO (□), DPDPE (▲), dermenkephalin (▽), and (L-Met^2) dermenkephalin (●) and inhibition of [^{3}H]dermorphin specific binding (0.5 nM) to rat brain membranes by increasing concentrations of unlabeled dermorphin (■), dermenkephalin (○), and (L-Met^2) dermenkephalin (◆) . The percentage of specific binding was calculated as 100 × $(B_s - B_n)/(Bo - Bn)$, where B_s and B_o are, respectively, the amount bound in the presence or the absence of competing compound and B_n is the nonspecific binding, i.e., the binding in the presence of 1000 nM unlabeled DAGO or dermorphin. The *solid lines* are theoretical fits to a simple binding isotherm determined by nonlinear least squares regression analysis. The data shown are for a single representative experiment. Values for IC_{50} were determined by regression analysis based upon two to four independent experiments carried out in duplicate.

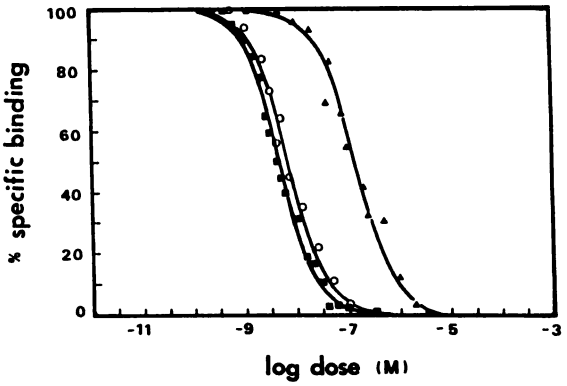

Fig. 3. Inhibition of [^{3}H]DPDPE specific binding (1 nM) to rat brain membranes (0.74 mg/ml membrane proteins) by increasing concentrations of unlabeled DAGO (▲), DPDPE (■), and dermenkephalin (○). The percentage of specific binding was calculated as $100 \times (B_s - B_n)/(B_o - B_n)$, in which B_s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

macological profiles of dermenkephalin and (L-Met^2)dermenkephalin on isolated tissues were evaluated on MVD and GPI, respectively (16, 17). Four different concentrations of each ligand (six assays for each) were tested for inhibition of electrically induced contractions. IC_{50} values were determined by regression analysis and Met-enkephalin was used in each assay as internal standard, according to the method of Kosterlitz *et al.* (18).

Results

The opioid receptor binding profiles of dermenkephalin and (L-Met^2)dermenkephalin for the μ and the δ sites of rat brain were determined by competition experiments using [^{3}H]DAGO (19) or [^{3}H]dermorphin (9) as prototypical μ receptor ligands and [^{3}H] DPDPE as δ-selective ligand (20, 21). As expected (22), our results in whole brain indicated that unlabeled DAGO and dermorphin are, respectively, 416 and 600 times more potent than unlabeled DPDPE in displacing bound [^{3}H]DAGO (Figs. 2 and 3). The inverse holds for displacement of the prototypical δ receptor ligand [^{3}H]DPDPE from rat brain membrane preparations (Figs. 2 and 3). These findings are in accordance with the reported high selectivity of DAGO, and DPDPE for the μ and the δ opioid receptors, respectively (9, 22). The K_i values of DAGO dermorphin and DPDPE towards μ and δ sites, respectively, together with the corresponding selectivity ratios, $K_{i(\mu)}/K_{i(\delta)}$ are reported in Table 1.

An unambiguous situation was encountered when the ability of dermenkephalin to displace these highly selective ^{3}H-opioids was examined. As shown in Fig. 3, dermenkephalin competitively inhibited the high affinity specific [^{3}H]DPDPE binding in a concentration-dependent manner, with a 50% inhibitory concentration of 6.9 nM and a quasi-Hill coefficient close to unity (Table 1). The K_i of dermenkephalin towards the δ site labeled by [^{3}H]DPDPE was 3.15 nM, a value close to that determined for unlabeled DPDPE under similar experimental conditions. In contrast, the highly μ-selective opioid peptides [^{3}H]DAGO and [^{3}H]dermorphin were displaced with very low potency by dermenkephalin (Fig. 2; Table 1), with K_i values close to those evaluated with unlabeled DPDPE under similar conditions (Fig. 2; Table I). Note that the displacement curves for dermenkephalin and the tested selective ligands could all be fit to a simple competitive model assuming only one homogeneous population of binding sites. No evidence for a more complicated model was observed.

In order to further characterize the selectivity pattern of dermenkephalin, its cross-reactivity towards the κ opioid receptors was investigated using [^{3}H]EKC, a nonselective κ ligand (23). This was performed either directly in preparations of guinea pig cerebellum membranes (24) or in the presence of 100 nM DAGO and 100 nM DPDPE in preparations of rat brain membranes (25). Whereas unlabeled EKC competed for the binding of [^{3}H]EKC with a K_i of 2 nM in homogenates of guinea pig cerebellum, dermenkephalin and (L-Met^2)dermenkephalin were found to be virtually inactive (K_i > 25000 nM) (Table I). Similar results were obtained in preparations of rat brain membranes in the presence of μ and δ blockers (data not shown). As shown in Table I, the K_i values of dermenkephalin are 367–619 and 3.15 nM, respectively, for μ and δ binding sites,

TABLE 1

Potencies of opioid ligands in inhibiting the binding at the μ sites (0.5 nM [^{3}H]DAGO, or 0.5 nM [^{3}H]dermorphin), the δ site (1 nM) [^{3}H]DPDPE) and the κ sites (1.2 nM) [^{3}H]EKC in homogenates of rat brain or guinea pig cerebellum, at 24 in 50 mM Tris·HCl buffer, pH 7.4

Because no determination of actual free radioligand concentration was done, K_i values reported in this table are possibly inaccurate (30).

Ligand	[^{3}H]DAGO		[^{3}H]Dermorphin		[^{3}H]DPDPE		[^{3}H]EKC[a]		$\frac{K_{i(\delta)}{}^b}{K_{i(\mu)}}$
	K_i[c]	n_H[d]	K_i	n_H	K_i	n_H	K_i	n_H	
	nM		nM		nM		nM		
DAGO	1.06 (0.09)	0.89 (0.04)	ND[e]		64.7 (4.70)	1.13 (0.06)	>20,000		61.0
Dermorphin[f]	0.70 (0.02)	1.11 (0.03)	0.73 (0.02)	0.97 (0.03)	61.7 (4.1)	1.17 (0.08)	>10,000		88.1
DPDPE	421 (31)	0.98 (0.04)	595[f] (41)	0.89 (0.03)	2.15 (0.08)	0.98 (0.03)	>20,000		0.0051 (0.0036)[g]
EKC	ND		ND		ND		1.98 (0.08)	1.14 (0.05)	
Dermenkephalin	367 (23)	1.02 (0.07)	619 (56)	1.11 (0.10)	3.15 (0.20)	1.12 (0.07)	>25,000		0.0085 (0.0050)
(L-Met^2)Dermenkephalin	3,347 (106)	1.10 (0.04)	3374 (151)	0.91 (0.04)	>5000		>25,000		

[a] Inhibition of [^{3}H]EKC binding in homogenates of guinea pig cerebellum.
[b] The selectivity ratio at the δ site is expressed as the ratio K_i for δ versus [^{3}H]DPDPE)/K_i for μ versus [^{3}H]DAGO).
[c] The inhibitory constant K_i was calculated from IC_{50} values using the Cheng and Prussof equation (13). Reported values are the mean of two to four experiments carried out in duplicate. Values in parentheses are standard errors.
[d] Quasi-Hill coefficient. Values in parentheses are standard errors.
[e] Not determined in that study.
[f] Taken from Ref. 9.
[g] Values in parentheses are selectivity ratios at the δ site expressed as the ratio K_i for δ (versus [^{3}H]DPDPE)/K_i for μ (versus [^{3}H]dermorphin).

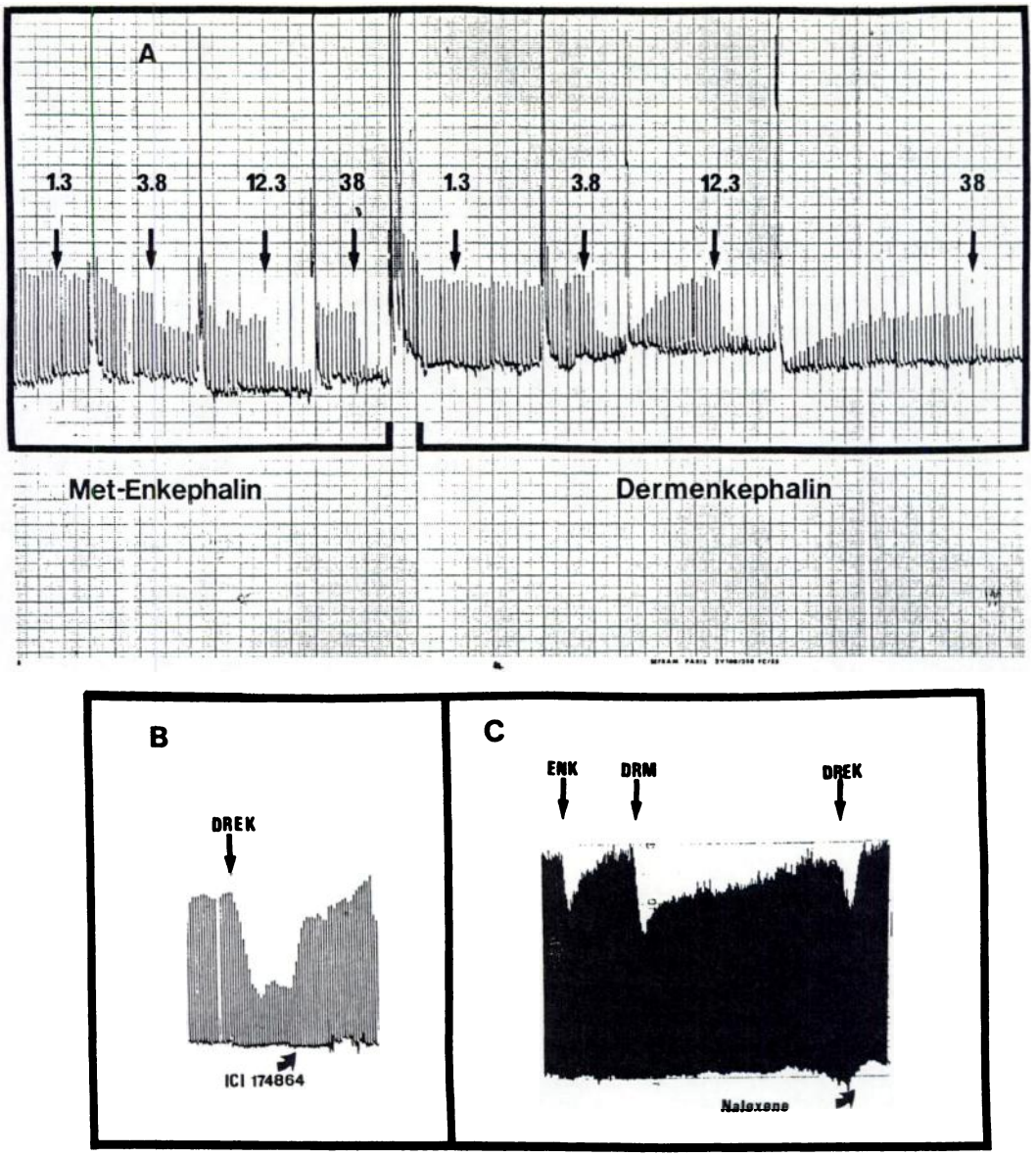

Fig. 4. Effects of some opioid agonists and antagonists on the electrically evoked contractions of the isolated MVD and GPI. A, Inhibition of electrically evoked contractions of the MVD incubated with graded concentrations (nM) (*arrows*) of Met-enkephalin and dermenkephalin and duration of recovery upon washing with fresh bathing solution. Recovery was rapid for Met-enkephalin and slowest for dermenkephalin. B, Antagonism by ICI 174,864 (100 nM) in the MVD against the agonist action of 4 nM dermenkephalin (*DREK*). C, Inhibition and reversal by naloxone (30 nM) of electrically evoked contractions of the GPI incubated with 100 nM enkephalin (*ENK*), 10 nM dermorphin (*DRM*), and 500 nM dermenkephalin (*DREK*).

TABLE 2

Inhibitory potencies (IC_{50}) of opioid peptides on the electrically evoked contractions of MVD and GPI

IC_{50} is the concentration that supresses the electrically stimulated muscle contractions by 50%. Reported IC_{50} values are the mean of six experiments. Values in parentheses are standard errors.

Ligand	IC_{50}		IC_{50} (MVD)/IC_{50} (GPI)
	MVD	GPI	
	nM		
Met-Enkephalin	7.80 (0.87)	230 (27)	0.0339
Dermenkephalin	2.08 (0.20)	3475 (272)	0.00059
Dermorphin[a]	29.0 (3.35)	3.30 (0.22)	8.800
DPDPE[b]	2.19 (0.30)	6930 (124)	0.00031
DTLET[c]	0.15 (0.01)	460 (46)	0.00032

[a] Taken from Ref. 4.
[b] Taken from Ref. 20.
[c] Taken from Ref. 28.

indicating a selectivity ration $K_{i(\delta)}/K_{i(\mu)} = 0.0085–0.0050$. Under similar experimental conditions, DPDPE, a prototypical δ ligand, exhibited a selectivity index ranging from 0.0051 (versus [^{3}H]DAGO as μ ligand) to 0.0036 (versus [^{3}H]dermorphin as μ ligand) (Table 1). Thus, the selectivity and binding affinity of dermenkephalin for the δ receptor are close to those exhibited by one of the purest δ probes available to date (20, 21, 23). On the other hand, (L-Met^2)dermenkephalin was found to be virtually inactive in displacing both μ- and δ-selective probes (Figs. 2 and 3; Table 1), a situation already encountered with (L-Ala^2)dermorphin (6, 7).

The opioid receptor selectivity of dermenkephalin was further evaluated in the MVD and GPI assays, using Met-enkephalin as internal standard. As shown in Fig. 4, dermenkephalin inhibited the electrically evoked contractions of GPI and MVD preparations. These inhibitions are opioid in nature, inasmuch as they are reversed by naloxone. Further support for the proposal that dermenkephalin acts as a δ agonist was obtained through the use of the selective δ antagonist ICI 174,864. As shown in Fig. 4, 100 nM ICI 174,864 fully reversed the inhibition of the electrically evoked contractions of MVD preparations induced by 4 nM dermenkephalin. (L-Met^2)Dermenkephalin was inactive in these assays (data not shown). The ratio of the IC_{50} value in the MVD assay to that of the GPI assay (Table

2) can be taken as an index of the selectivity of dermenkephalin for the δ versus the μ receptor (18). Based on this ratio, dermenkephalin turns out to be among the most selective ligands tested, having an IC_{50} ratio of 0.0005, compared with 0.034, 0.0003–0.00136 (20, 27), and 0.0003–0.0058 (20, 28), respectively, for Met-enkephalin and the highly selective δ ligands DPDPE and DTLET. (Table 2). Moreover, it is clear from the results in Table 2 that dermenkephalin is more potent than Met-enkephalin in inhibiting the electrically induced contractions of MVD, a finding consistent with a high δ selectivity and agonist potency. Finally, the potency of morphine, dermorphin, dermenkephalin, and (L-Met^2)dermenkephalin in inducing analgesia after intracerebroventricular administration to mice was evaluated (data not shown). Whereas dermorphin was the most active peptide, being 700 time more potent than morphine [AD_{50} = 0.0060 (95% confidence limit, 0.0044–0.0086) and 4.2 (95% confidence limit, 2.7–6.6) nmol/mouse, respectively], 100 nmol of dermenkephalin had no effect on the escape latency.

Discussion

Collectively, results from the present study show, on the basis of both binding and pharmacological assays *in vitro*, that dermenkephalin is one of the most δ-selective ligands reported to date (20, 21, 29). Dermenkephalin combines a high receptor selectivity with a high potency in inhibiting both the electrically evoked contractions of the rat deferens and the binding of fully δ-selective ligands to the rat brain membrane preparations. Because dermenkephalin has a primary structure different from that of all the hitherto known naturally occuring and synthetic opioid peptides, it should be a useful tool for identifying molecular and conformational determinants of high affinity binding of opioid peptides to the δ receptor. The finding that (L-Met^2)dermenkephalin was found to be devoid of opioid activity is to be interpreted with caution, because the succeptibility of this peptide to proteolysis during *in vitro* binding or pharmacological assays was not evaluated. As highlighted by the enkephalins, much of the potency difference between dermenkephalin and its L-Met version could be due to greater degradation of the latter. Further analyses conducted with a combination of protease inhibitors (bestatin, captopril, amastatin, thiorphan) are, therefore, needed to evaluate this proposal.

Of the utmost interest is the possible occurence of dermenkephalin as a natural processing product of prodermorphin. Based on the biosynthesis of dermorphin, production of dermenkephalin would require both that cleavage/processing of prodermorphin occurs N- and C-terminal to the dermenkephalin sequence and that an isomerase able to recognize the Tyr-D-X-Phe sequence common to dermorphin and dermenkephalin is present. The use of oligonucleotide probes and/or highly specific dermenkephalin antibodies might be helpful in answering these questions. If this peptide is actually produced *in vivo*, prodermorphin would, thus, provide a surprising example of an opioid biosynthetic precursor that could simultaneously generate two D-amino acid-containing peptides exhibiting a high affinity and a strict selectivity for two distinct opioid receptors.

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