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DEHYDRO-ENKEPHALINS.IV. DISCRIMINATIVE RECOGNITION OF DELTA AND MU OPIATE RECEPTORS BY ENKEPHALIN ANALOGS

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We have studied the receptor binding activities of C-terminal free and amidated enkephalins with and without the dehydrophenylalanine[4] residue. For the selective labeling of so-called δ and μ opiate receptors, specific tracers were used at low concentrations in rat brain membranes and neuroblastoma cells containing pure δ receptors. C-Terminal free enkephalins are five to eight times more potent in the assay for δ receptors than in μ assay, while the amides are almost equipotent in both assays. The presence of a C-terminal carboxyl group is a determining factor for selective activity. [D-Ala^2, ΔPhe^4, Met^5]-enkephalin amide is very potent in all of the binding assays examined, and, in particular, twice as active as the saturated amide and the C-terminal free enkephalin in the δ assay. We suggest that the steric arrangement of the dehydrophenylalanine residue in position 4 is very important to the enhanced interaction with the δ receptors.

INTRODUCTION

Since the presence of multiple opiate receptors in the brain was proposed[1,2] and the so-called δ and μ opiate receptors have been well characterized,[2,3,4] opioid peptides have been placed into at least three different classes with δ, μ, and δ,μ-intermediate receptor activity. To elucidate the nature of these receptors, it is important to examine the structures of these peptides for their specific interactions with each receptor. Although several peptides have been found to be preferential ligands for δ[5,6] or μ[7] receptors in recent years, the structural factors for their receptor interactions have not been clearly elucidated.

We have recently synthesized a systematically unsaturated series of enkephalin analogs, dehydro-enkephalins,[8] which contain

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α,β-dehydroamino acids and C-terminal carboxyl groups. We have shown that incorporation of a dehydroamino acid residue is an effective way to obtain enkephalin analogs with full receptor activity which are resistant to enzymatic hydrolysis without changing their receptor selectivity.[9] A dehydro-enkephalin H-Tyr-D-Ala-Gly-ΔPhe-Leu-OH† showed high activity and selectivity for δ receptors, suggesting that the fixed phenyl ring of ΔPhe^4 in the Z-configuration may effect the interaction with the receptor. On the other hand, the amidated analog H-Tyr-D-Ala Gly-ΔPhe-Met-NH_2 had five times the activity of the corresponding saturated peptide in the electrically stimulated guinea pig ileum (GPI) μ assay.[10] Although the blocking of the C-terminus by amidation or esterification invariably yields peptides with μ or δ,μ-intermediate activity,[11] we were interested in the receptor binding activity of this amidated dehydro-enkephalin with a view to examining the effects of amidation and incorporation of an α,β-dehydroamino acid on the interaction of the peptide with each receptor.

As labeled ligands, ^{125}I-labeled enkephalins and tritiated naloxone were utilized in rat brain membrane preparations, and tritiated enkephalin in neuroblastoma cell lines. Pairs of C-terminal free and amidated enkephalins, namely Leu^5, Met^5, and ΔPhe^4-enkephalins were examined in each specific receptor assay.

MATERIAL AND METHODS

Peptides

The synthesis of ΔPhe^4-enkephalins were carried out by conventional solution methods and reported elsewhere.[8,10] All other peptides were purchased from Peninsula Laboratories, Inc. (San Carlos, CA).

Radio Ligand Binding Assays

Receptor binding assays using rat brain membrane preparations were performed essentially as described.[12] Incubations were carried out for 1 hr at 25°C in 50 mM Tris·HCl (pH 7.5) buffer containing bacitracin (100 μg/ml). Monoiodinated[13] [^{125}I]-[D-Ala^2, D-Leu^5]-enkephalin (^{125}I-DADLE) and [^{125}I]-[D-Ala^2, Leu^5]-enkephalin amide (^{125}I-DALEA) were used at a final concentration of 0.05 nM, and [^3H] Naloxone (^3H-NAL, 50.2 Ci/mmol, New England Nuclear) at 0.15 nM. An assay utilizing neuroblastoma cells (NG108-15) and [^3H]-[D-Ala^2, D-Leu^5]-

†Abbreviations: Δ, dehydro (α,β-unsaturated); ^3H- or ^{125}I-DADLE, [^3H]- or [^{125}I]-[D-Ala^2, D-Leu^5]-enkephalin; ^{125}I-DALEA, [^{125}I]-[D-Ala^2, Leu^5]-enkephalin amide; ^3H-NAL, [^3H]naloxone; DALE, DAME, DALEA, DAMEA, ΔPhe^4-OH and ΔPhe^4-NH_2, refer to the amino acid sequences of these enkephalins in Table 1.

enkephalin (^{3}H-DADLE, 40 Ci/mmol, New England Nuclear) was carried out according to the procedure of Chang et al.[14]

Dose-response curves were constructed utilizing eight or ten dose levels, in duplicate. Results were analyzed by the computer program "ALLFIT", to construct the least-squares estimates of the logistic curves relating binding of labeled ligand to concentrations of unlabeled ligand.[15] Non -specific binding obtained for each tracer in the presence of the corresponding unlabeled ligand, was subtracted from all the data. They were 20-30% of total bound counts for ^{125}-I-DADLE and ^{125}I-DALEA, 20% for ^{3}H-NAL in rat brain, and 5% for ^{3}H-DADLE in NG108-15 neuroblastoma cells.

RESULTS

Table 1 shows all the receptor binding potencies. Very low concentrations of radio-labeled C-terminal free enkephalin, ^{125}I-DADLE and alkaloid ^{3}H-NAL, label almost selectively δ and μ receptors, respectively, and allow measurement of the selective affinities of the peptides for opiate binding sites in rat brain membrane, under similar conditions.[3] In ^{125}I-DADLE assay, C-terminal free [D-Ala^2, Leu^5 or Met^5]-enkephalins (DALE or DAME) are slightly more potent than their amidated analogs (DALEA or DAMEA). In ^{3}H-NAL or μ assay, the amides are almost equipotent to δ assay, while C-terminal free peptides show a decreased potency by a factor of five to eight. An assay using ^{3}H-DADLE in neuroblastoma cells (NG108-15) which contains only δ receptors[14,16] gives nearly the same results as ^{125}I-DADLE assay in rat brain.

Table 1. Receptor Binding Activity of Enkephalin Analogs.

Enkephalin	Amino acid residue[a] 4	5	C-terminus	ED_{50} (nM)[b] ^{125}I-DADLE (rat brain)	^{3}H-NAL (rat brain)	^{3}H-DADLE (cells)	^{125}I-DALEA (rat brain)
DALE	Phe	Leu	COOH	0.81(±0.02)	6.76(±1.09)	1.04(±0.07)	2.95(±0.45)
DAME	Phe	Met	COOH	1.21(±0.22)	6.06(±0.89)	0.80(±0.05)	3.12(±0.44)
ΔPhe^4-OH	ΔPhe	Leu	COOH	1.27(±0.16)	10.1 (±1.66)	1.45(±0.10)	2.51(±0.14)
DALEA	Phe	Leu	$CONH_2$	1.46(±0.37)	1.14(±0.19)	1.41(±0.10)	1.13(±0.18)
DAMEA	Phe	Met	$CONH_2$	1.64(±0.19)	1.97(±0.29)	1.48(±0.10)	0.73(±0.10)
ΔPhe^4-NH_2	ΔPhe	Met	$CONH_2$	0.61(±0.07)	1.46(±0.25)	0.75(±0.04)	0.49(±0.07)

[a] All the peptides have the same sequence Tyr^1-D-Ala^2-Gly^3.

[b] ED_{50}'s show the dose which produces a 50% inhibition of binding of each tracer, and the values are nM ± s.e.

This confirms that in the latter assay we are able to see only δ receptor binding activity as described.[16]

Radio-labeled [^{125}I]-[D-Ala^2, Leu^5]-enkephalin amide (^{125}I-DALEA) gives values intermediate between δ and μ assays, and so it may be considered as δ,μ-combined assay. This is because the amidated enkephalin can bind well to both δ and μ receptors as described before[17] (Table 1). Note that both ^{125}I-DALEA and ^{125}I-DADLE were used as tracers at a final concentration of 0.05 nM, which is more than 10 times lower than their affinities (Kd's) for the receptor. So the differences in ED_{50} of various peptides in these two assays mainly reflect differences in receptor affinities.

[D-Ala^2, ΔPhe^4, Leu^5]-enkephalin (ΔPhe^4-OH) is almost as active as the corresponding saturated analog.[9] Surprisingly, amidated [D-Ala^2, ΔPhe^4, Met^5]-enkephalin amide (ΔPhe^4-NH_2) is the most active compound assayed.

The ability of each peptide to discriminate between δ and μ receptors is expressed as a ratio of the ED_{50} using ^{3}H-NAL versus ED_{50} using ^{125}I-DADLE in parallel binding assays utilizing rat brain membrane preparations (Fig. 1). C-Terminal free enkephalins show a high selectivity for δ receptors (ratio = 5-8), and the amides are equipotent against δ and μ sites with selectivity nearly one. It is clear, however, that ΔPhe^4-NH_2 enkephalin has a shifted value towards δ site with a δ selectivity which is twice that of the saturated analog. Fig. 2 shows the potency of enkephalins in two assays;

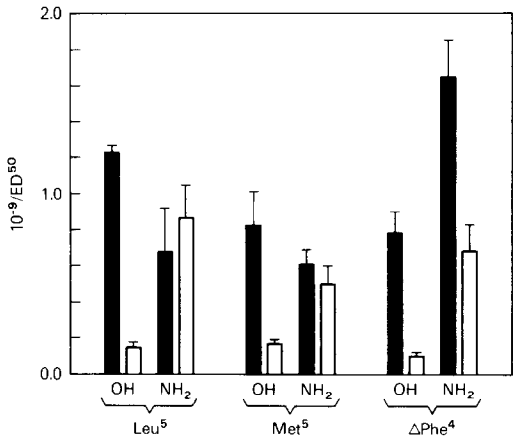

Figure 1. Relative affinity ($10^{-9}/ED_{50}$; nM^{-1}) of enkephalins for the δ and μ opiate receptors: the δ activity (solid bars) using ^{125}I-DADLE, and μ activity (empty bars) using ^{3}H-NAL in rat brain membranes.

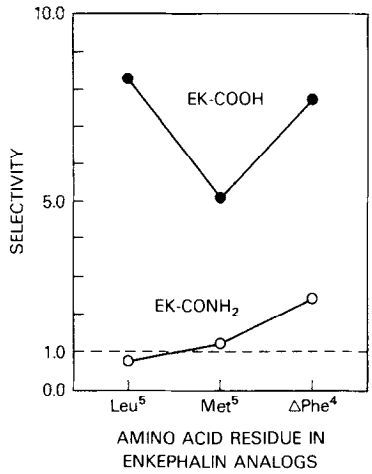

Figure 2. Selectivity ratio of C-terminal free enkephalins and amidated enkephalins for the δ and μ opiate receptors.

it is easy to estimate and compare both activity and selectivity simultaneously. All C-terminal free enkephalins including the ΔPhe^4-peptide display a similar pattern. The saturated amides DALEA and DAMEA also show similar profiles, but the amidated ΔPhe^4 enkephalin is exceptional in its distinctly higher potency for the δ receptors. This is a significant finding and may indicate either that the δ receptor is more lipophilic than the μ site or that the modified conformation of the $\Delta^z Phe^4$ peptide (possible β-turn) fits the δ receptor preferentially.

DISCUSSION

Rónai et al.,[18] using the mouse vas deferens (MVD) for δ and GPI for μ receptors, have showed that the acidic character of the C-terminus of enkephalin is a precondition for one enkephalin-like property, namely δ activity. Amidated peptides invariably lose their agonist activity in the MVD δ assay. In the present receptor binding studies, results also clearly show that the existence of a C-terminal free carboxyl group is a determinant for δ activity and selectivity, but the major effect is the loss of μ activity by the C-terminal free enkephalin (Table 1). DALE and DAME are only slightly more potent than the amides DALEA and DAMEA in δ assays (^{125}I-DADLE in rat brain and ^{3}H-DADLE in neuroblastoma cells), but they are 5 to 8 times less potent in μ assay (^{3}H-NAL in rat brain (Table 1, Fig. 2). On the other hand, the amides are as active in the μ assay as

in the δ assay (Table 1, Figs. 1,2). A similar result has been reported by Kosterlitz et al.[17]

In summary, although both in vitro assays show that amidated enkephalins shift selectivity towards the μ receptor, the mechanism of this shift is different in the two assays. For the muscle assays it is mainly due to a decrease in δ activity of the amides, while for the receptor binding assays it is due to a decrease in the μ activity of C-terminal free peptides. This difference cannot be explained readily at the moment, however,the possibility that in the muscle assays a degradation of the carboxyl-free peptides may occur, or that in the MVD assay both μ and δ receptors are present[2] may account for the difference.

[D-Ala^2, ΔPhe^4, Met^5]-enkephalin amide displayed an unexpectedly high δ activity, which is almost twice that of the δ-selective [D-Ala^2, ΔPhe^4, Leu^5]-enkephalin. The differences in the C-terminal amino acids and the free carboxyl vs. amide structures cannot easily explain this phenomenon since there is no such large difference between the saturated Leu^5 and Met^5-enkephalins (Table 1). Therefore, it appears that the high δ activity of amidated ΔPhe^4-NH_2 enkephalin is due to the ΔPhe^4 residue. α,β-Dehydrogenation of a peptide backbone confers increased rigidity and hydrophobicity on the peptide. The ΔPhe^4 residue of the dehydro enkephalin has the Z-configuration; i.e., the phenyl group is cis to the nitrogen atom of ΔPhe^4, and so is held rigidly towards the amino end of the peptide chain.[10] Since the C-terminal free ΔPhe^4-OH enkephalin shows high selectivity for the δ site, having almost the same potency as the saturated analog[9] (Table 1), it is unlikely that it is the increased hydrophobicity in position 4 which facilitates selective interaction with the receptors. Roques et al.[19] have reported that a complete loss of activity toward the δ receptors (MVD) was found when the phenyl ring was replaced by an aliphatic hydrophobic moiety. Consequently, it is clear that the presence of the phenyl ring in position 4 is important for δ activity, and the present study strongly suggests that the Z-configuration places the phenyl ring in a very favorable position for δ receptor interaction. The synthesis of ΔPhe^4 enkephalin which has the E-configuration is now in progress and further elucidation of this point will follow.

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