Note

Thiol-containing Enkephalins for Exploration of the Essential Thiol Group in Opiate Receptors

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Stereospecific binding of opiate agonists and antagonists is effectively inhibited by thiol reagents.^{1~3)} Simon *et al.*¹⁾ have suggested the presence of an essential SH group near the binding site of the opiate receptor. Although Hazum *et al.*³⁾ have postulated a receptor microaggregation process in which the SH group would participate in the receptor dimerization, no role is known for this essential SH group in the molecular mechanism of opiate receptors. In this study, to explore such SH groups, we have prepared two thiol-containing enkephalins which have SH groups within the peptide backbone; namely, [D-Ala², Cysteamine⁵]enkephalin (**1**) and [D-Ala², Leu⁵, Cysteamine⁶]enkephalin (**2**).

For the synthesis of thiol-containing enkephalin analogs, a cysteamine residue was incorporated as its oxidized form, cystamine. Cystamine was coupled with Boc-Phe-OH or Boc-Phe-Leu-OH, and the resulting dimeric peptides were elongated with Boc-Tyr-D-Ala-Gly-OH.

Dimeric tetra- and pentapeptides crosslinked by cystamine were liberated with trifluoroacetic acid, and reduced to their monomeric analogs by zinc dust-HCl. They were purified by gel-filtration (Sephadex G-15, 30% AcOH), HPLC (Whatman Partisil ODS, 60% MeOH-0.1 M NH₄OAc), and cation-exchange chromatography (CM-cellulose, 30% AcOH). Their homogeneity was examined by thin-layer chromatography, paper electrophoresis, amino acid analysis, and elemental analysis.

All peptides were evaluated for their activity in two different radioligand assay systems, essentially as described elsewhere.⁴⁾ [³H]DADLE (40 Ci/mmol) and [³H]DAGO (30 Ci/mmol) purchased from New England Nuclear, Boston, Mass, were used as labelled ligands specific for delta and mu receptors, respectively, in rat brain membrane preparations at a final concentration of 0.25 nM. Incubations were done at 25°C for 60 min in 50 mM Tris-HCl buffer (pH 7.4) containing bestatin (1 μM) and bacitracin (100 μg/ml) as inhibitors of enkephalin-degradating enzymes. Dose-response curves were constructed using 6~10 dose levels and analyzed by computer programs to estimate the IC₅₀ value, the dose which produces a 50% inhibition of binding of each labelled ligand.

Table I shows the results of the receptor binding assays. Use of [³H]DADLE as labeled ligand is thought to reflect delta binding activity, while [³H]DAGO was used as the labeled ligand for mu receptors.⁵⁾ the propriety of binding assays was easily proven, since the unlabeled ligands of DADLE and DAGO showed an appropriate selectivity with high specificity for delta and mu receptors, respectively (Table I). When thiol-containing enkephalins **1** and **2** were examined in this assay system, both analogs showed high potencies with the IC₅₀ values of 3.02~19.2 nM (Table I). This indicates that the SH group in the C-terminal portions of enkephalin peptides can tolerate the interaction between peptide and receptors.

For mu receptors, **1** and **2** had almost the same affinity. Although their IC₅₀ values (5.25 nM and 5.86 nM, respectively) are slightly lower than that (1.15 nM) of mu-selective DAGO, those should be interpreted to be fairly high. For delta receptors, **2** showed significantly less

TABLE I. RECEPTOR BINDING AFFINITIES OF ENKEPHALIN ANALOGS

Enkephalin ^a	IC ₅₀ (nM)		IC ₅₀ Ratio [³ H]DAGO/[³ H]DADLE as δ-selectivity
	[³ H]DADLE	[³ H]DAGO	
[D-Ala ² ,Cta ⁶]Enk (1)	3.02	5.25	1.74
[D-Ala ² ,Cta ⁵]Enk (2)	19.2	5.86	0.30
DADLE ^b	1.70	7.59	4.46
DAGO ^c	89.1	1.15	0.013

^a Cysteamine was abbreviated as Cta in this table.

^b [D-Ala²,D-Leu⁵]enkephalin.

^c [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin.

affinity than delta-selective DADLE, while peptide **1** showed a fairly high receptor affinity.

Some possible structural elements in the opiate receptors⁶⁾ such as cationic site, metal-ion site and the SH group may interact with the C-terminal portion of enkephalins. If the opiate receptor contains a metal-ion site, the C-terminal thiol of enkephalin, especially [D-Ala², Cysteamine⁵]enkephalin which has exactly the same peptide backbone length as the usual enkephalin pentapeptides, would interact strongly with this site. However, binding characteristics of thiol-containing enkephalin analogs in this study suggests that no metal-ion site is present in the binding site, since these analogs did not have activity enhanced as much as expected from that of the standard enkephalins (Table I). Nevertheless, a fairly high potency of thiol-containing enkephalins may reveal the interaction of these peptides with the opiate receptor to produce a probable disulfide bridging between peptide and receptor. If this is the case, formation of the S-S bond must be very slow because of a requirement of oxidative conditions.

This study provided the first enkephalin analogs having SH groups in the peptide backbone, with high receptor affinity. The binding results further enforce us to design

and synthesize an analog which has a functional and activated SH-reacting group to capture chemically the essential SH group for elucidation of its role in the molecular mechanism.

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