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Conformationally restricted analogs of somatostatin with high μ-opiate receptor specificity

(rat brain receptor binding/peptide hormones/enkephalin)

JOHN T. PELTON*, KAROLY GULYA†‡, VICTOR J. HRUBY*§, SUE PIPER DUCKLES†, AND HENRY I. YAMAMURA†

*Department of Chemistry, University of Arizona, Tucson, AZ 85721; and †Department of Pharmacology, University of Arizona Health Sciences Center, Tucson, AZ 85724

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ABSTRACT **A series of cyclic, conformationally restricted analogs of somatostatin have been prepared and tested for their ability to inhibit the binding of [^{3}H]naloxone and [D-Ala^2, D-Leu^5][^{3}H]enkephalin to rat brain membranes. The most potent analog, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH$_2$ where Pen is penicillamine in [D-Phe^5, Cys^6, Tyr^7, D-Trp^8, Pen^{11}]somatostatin-(5–12)-octapeptide amide, exhibited high affinity for μ-opiate receptors (IC_{50} value of [^{3}H]naloxone = 3.5 nM), being 7800 times more potent than somatostatin. The cyclic octapeptide also displayed high μ-opiate receptor selectivity with an IC_{50} ([D-Ala^2, D-Leu^5]enkephalin)/IC_{50} (naloxone) ratio of 271. The high affinity and selectivity of the somatostatin analog for μ-opiate receptors may be of use in examining the physiological role(s) of the μ-opiate receptor.**

The cyclic tetradecapeptide somatostatin is known to interact with a variety of receptor systems over a wide concentration range (1). High-affinity (low dissociation constant) binding with a receptor system is generally thought to have physiological significance, but when the affinity is weak (micromolar), the physiological significance is not clear. The weak affinity that somatostatin displays for the opiate receptor is one such example. It has been reported, for instance, that high concentrations (high micromolar) of somatostatin can inhibit the binding of [^{3}H]naloxone and [D-Ala^2, D-Leu^5][^{3}H]enkephalin ([^{3}H]DADLE) to rat brain homogenates as well as give rise to an *in vivo* analgesic response in mice (2, 3). Recently, it has been reported that the somatostatin analog [D-Phe^5, Cys^6, D-Trp^8, Cys^{11}]somatostatinol-(5–12)-octapeptide, which we refer to as D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol), with an IC_{50} value of 38 nM at the μ-opiate receptor, was capable of antagonizing the excitatory effects of the stable enkephalin analog [D-Ala^2, MePhe^4, -NHCH(CH$_2$OH)-(CH$_2$)$_2$-S(O)CH$_3^5$]enkephalin in electrophysiological experiments (4). Our interest in somatostatin stems from the reported antagonist activity of this analog to opiates as well as the apparent lack of structural similarity of somatostatin and analogs to either the rigid opiates or the enkephalin compounds.

We have prepared a number of conformationally restricted analogs of somatostatin in order to probe the structural and conformational features important for somatostatin's activity at opiate receptors. These studies have resulted in the development of the penicillamine (Pen)-containing analog [D-Phe^5, Cys^6, Tyr^7, D-Trp^8, Pen^{11}]somatostatin-(5–12)-octapeptide amide, which we refer to as D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH$_2$ or [Cys^2, Tyr^3, Pen^7]-octapeptide amide and which displays high μ-opiate receptor affinity and selectivity in the rat brain radioreceptor assay (Fig. 1).

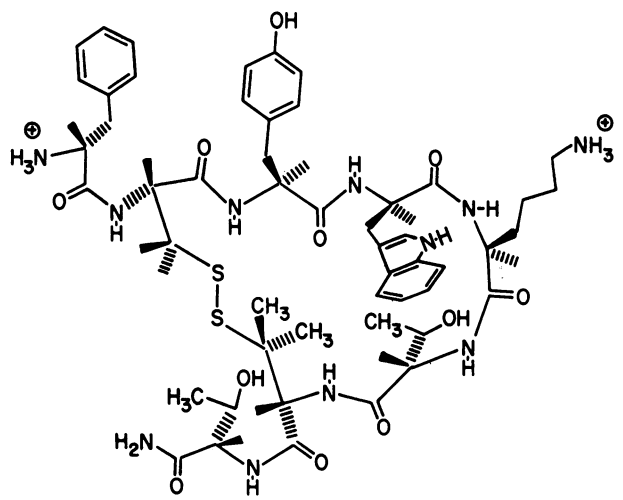

FIG. 1. Structure of the most potent μ-opiate receptor-selective somatostatin analog, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH$_2$.

MATERIALS AND METHODS

Somatostatin was purchased from Vega Biotechnologies. The analog CGP 23,996 (des-Ala^1, Gly^2-[desamino-Cys^3, Tyr^{11}]-3,14-dicarbasomatostatin) was a generous gift from CIBA–Geigy. Amino acid analyses were performed on a Beckman 120C amino acid analyzer. All optically active amino acids are of the L variety unless otherwise noted. Cysteine was determined as cysteic acid (5). Fast-atom-bombardment mass spectra (FABMS) were obtained on a Varian 311A spectrometer equipped with an Ion Tech Ltd source with xenon as the bombarding gas.

Analogs were synthesized by standard solid-phase synthetic techniques (6, 7). N^{α}-*t*-Butyloxycarbonyl (Boc)-protected amino acids were used throughout the syntheses and were prepared in the usual manner (8, 9). For the preparation of peptides with a COOH-terminal carboxylic acid group, N^{α}-*t*-Boc-*O*-Bzl-L-threonine (Bzl = benzyl) was attached to chloromethylated copoly(styrene-1% divinylbenzene) beads (Lab Systems, 0.71 milliequivalent of Cl per g of resin) by the method of Gisin (10). Carboxamide peptides were prepared with a *p*-methylbenzhydrylamine resin (threonine substitution, 0.35 mmol/g of resin) as described (7). Preformed symmetrical anhydrides were used in the coupling reactions (11), which were monitored by ninhydrin (12) and/or chloranil (13) tests and repeated as necessary. Peptides were de-

Abbreviations: [Cys^2, Cys^7]-octapeptide, [D-Phe^5, Cys^6, D-Trp^8, Cys^{11}]somatostatin-(5–12); Pen, penicillamine; FABMS, fast-atom-bombardment mass spectrometry; DADLE, [D-Ala^2, D-Leu^5]enkephalin.

‡Permanent address: Central Research Laboratory, Medical University, Szeged, Hungary.
§To whom reprint requests should be addressed.

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protected and removed from the resin with anhydrous liquid HF (10 ml/g of resin) containing 10% anisole. After cyclization with 0.01 M $K_3Fe(CN)_6$ at pH 8.4, the analogs were purified by gel filtration with 5% acetic acid on Sephadex G-15, SP-Sephadex cation-exchange chromatography, and partition chromatography (butanol/benzene/pyridine/0.1% acetic acid, 6:2:1:9, vol/vol) on Sephadex G-25 (block polymerizate). Purity was assessed by thin-layer chromatography in a minimum of five solvent systems, by paper electrophoresis at two different pH values, and by reversed-phase HPLC (Table 1). Integration of the HPLC chromatograms (λ = 214 nm) indicated purities were in excess of 95%. Amino acid analysis after acid hydrolysis gave the expected molar ratios (±7.0%) of the constituent amino acids. The ^1H and ^{13}C NMR spectra were obtained for each analog and were found to be consistent with the sequence and structure of the peptides. The $[M + H]^+$ molecular ions and fragmentation patterns obtained by FABMS were in agreement with the amino acid squence and composition of each analog.

Radioreceptor Assays. Adult Sprague–Dawley rats (150–200 g) were killed by decapitation. The brain was rapidly removed and homogenized (10% wt/vol) in 0.32 M sucrose in a glass homogenizer with a motor-driven Teflon pestle. The homogenate was centrifuged at 1000 × *g* for 10 min to remove the nuclear debris. The supernatant was then centrifuged at 43,000 × *g* for 10 min, and the resulting pellet was resuspended in 50 mM Tris (pH 7.4 at 25°C) containing 5 mM $MgCl_2$, bovine serum albumin at 2 mg/ml, and bacitracin at 20 μg/ml by using a Polytron homogenizer (15 sec, setting no. 5). The centrifugation and resuspension step was repeated once.

For all inhibition studies, rat brain plasma membranes (100 μl) were incubated at 25°C for 60 min in a total volume of 1.0 ml of 50 mM Tris buffer (as above) containing ≈220,000 dpm of ^{125}I-labeled CGP 23,996 (14), 1 nM [^3H]naloxone (42.3 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq), or 1 nM [^3H]DADLE (tritiated at the 3′ and 5′ positions of the tyrosine-1 residue; 43.5 Ci/mmol, New England Nuclear) and at least nine concentrations of our synthetic analogs. All incubations were done in duplicate, and each compound was tested at least five times. The concentration of test compounds was determined by quantitative amino acid analysis or from published molar extinction coefficients. Specific binding to somatostatin and to μ- and δ-opiate receptors was defined as the difference in the amounts of radioligands bound in the absence and presence of 1 μM somatostatin, 1 μM naltrexone, or 1 μM [Met^5]enkephalin, respectively. The data were analyzed using nonlinear least-squares regression analyses for the Apple II *Plus* computer. Computer programs were generously provided by Susan H. Yamamura.

RESULTS

The results of the opiate binding experiments are summarized in Table 2. All of the synthetic analogs inhibited the binding of both [^3H]naloxone and [^3H]DADLE to rat brain receptors, although there were significant differences when inhibitions of the two labeled ligands were compared. In agreement with a previous report (2), somatostatin had only weak activity in the rat brain radioreceptor assay, with IC_{50} values of 27 μM and 16 μM against [^3H]naloxone and [^3H]DADLE, respectively. The acyclic tetrapeptide Ac-Phe-D-Trp-Lys-Thr, similar to the presumed pharmacophore responsible for somatostatin's actions in inhibiting the release of growth hormone, glucagon, and insulin (15), also appears to be the active sequence for opiate activity. Indeed, the tetrapeptide was only slightly less potent than somatostatin at the μ-opiate receptor and somewhat more potent at δ-opiate receptors.

The conformationally restricted octapeptide analogs were generally more potent than somatostatin in both opiate binding assays. However, the substitution of penicillamine for cysteine at position 2 resulted in an analog with decreased receptor affinity relative to the same substitution at the penultimate 7 position. The receptor selectivity of D-Phe-Pen-Phe-D-Trp-Lys-Thr-Cys-Thr was also different from that of the other octapeptide compounds. Similar to somatostatin and the acyclic tetrapeptide analog, the [Pen^2, Cys^7]-peptide was slightly δ-opiate receptor-selective. All other analogs had modest to high degrees of μ-opiate receptor selectivity based on receptor binding experiments.

Increased conformational restriction imposed by the *gem*-dimethyl groups of penicillamine in the COOH-terminal region of the molecules resulted in analogs with increased receptor binding affinity and selectivity, as compared to the less-restricted [Cys^2, Cys^7]-octapeptide. A further increase in receptor affinity was obtained with the substitution of ty-

Table 1. Purity of synthetic peptides

Peptide	TLC*					Paper electrophoresis†, cm from origin		HPLC [k']‡				log P§
	I	II	III	IV	V	pH 2.2	pH 5.6	VI	VII	VIII	IX	
D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr	0.16	0.73	0.61	0.83	0.47	11.2	6.8	6.1	5.1	9.2	4.0	−0.77
D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr	0.23	0.73	0.64	0.85	0.58	11.2	6.8	6.9	6.0	11.5	5.4	−0.71
D-Phe-Pen-Phe-D-Trp-Lys-Thr-Cys-Thr	0.25	0.74	0.64	0.85	0.58	11.2	6.8	8.3	7.4	12.6	6.1	−0.22
D-Phe-Pen-Tyr-D-Trp-Lys-Thr-Cys-Thr	0.21	0.70	0.62	0.81	0.53	11.2	6.8	4.1	4.6	5.1	2.7	−0.69
D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr	0.23	0.68	0.63	0.82	0.53	11.3	6.8	3.0	3.8	4.9	2.2	−0.70
D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2	0.23	0.65	0.65	0.87	0.60	11.3	8.7	6.4	5.8	10.5	5.2	0.36
D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2	0.24	0.70	0.64	0.85	0.52	11.2	8.8	2.9	4.0	4.9	2.1	−0.20

*Baker 250-μm analytical silica gel glass plates were used. Solvent systems are: I, butanol/acetic acid/water, 4:1:5 (vol/vol) (upper phase); II, butanol, acetic acid/water/pyridine, 15:3:10:12 (vol/vol); III, butanol/acetic acid/water/pyridine, 6:1.2:4.8:6 (vol/vol); IV, isopropyl alcohol/ammonia/water, 3:1:1 (vol/vol); and V, butanol/pyridine/0.1% acetic acid, 5:3:11 (upper phase).

†Electrophoresis was at 450 V for 90 min at 4°C; peptides moved toward the cathode (reported as cm from the origin).

‡Capacity factor for the following systems: VI, Vydac 218TP15-16 C_{18} reversed-phase (RP) column (25 cm × 4.6 mm) with 0.1% trifluoroacetic acid/CH_3CN, 78:22 (vol/vol), at a flow rate of 2.5 ml/min; VII, Vydac 218TP5 C_4 RP column (25 cm × 4.6 mm) with 0.1% trifluoroacetic acid/CH_3CN, 77:23 (vol/vol), at a flow rate of 1.0 ml/min; VIII, Vydac 218TP15-16 C_{18} RP column with 0.1% hexafluorobutyric acid/CH_3CN, 74:26 (vol/vol), at a flow rate of 2.5 ml/min; IX, Zorbax ODS C_{18} RP column (25 cm × 4.6 mm) with 0.25 M TEAP buffer, pH 2.2/CH_3CN, 79:21 (vol/vol), at a flow rate of 1.0 ml/min. (TEAP buffer is triethylamine phosphate.) All peptides were monitored at λ = 214 nm.

§Partition coefficient for octanol/water, with the aqueous layer composed of 0.05 M Tris (pH 7.4), 0.005 M $MgCl_2$, and 0.15 M KCl.

Table 2. Potency of somatostatin and its analogs on [^{3}H]naloxone and [^{3}H]DADLE receptor binding to rat brain membranes

Peptide	[^{3}H]Naloxone		[^{3}H]DADLE		IC_{50} ratio DADLE/ naloxone
	IC_{50}, nM	Hill no.	IC_{50}, nM	Hill no.	
Somatostatin	27,400 ± 4,200	1.02	16,400 ± 8,500	1.09	0.60
D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2	3.5 ± 0.20	1.02	950 ± 210	0.33	271
D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2	9.9 ± 1.6	1.04	1,100 ± 120	0.33	111
D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr	290 ± 58	1.08	3,800 ± 610	0.72	13
D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr	930 ± 72	1.13	5,400	0.83	5.8
D-Phe-Pen-Tyr-D-Trp-Lys-Thr-Cys-Thr	470 ± 10	1.03	2,600 ± 410	0.58	5.5
D-Phe-Pen-Phe-D-Trp-Lys-Thr-Cys-Thr	61,000 ± 17,500	1.31	38,100 ± 3,000	1.02	0.62
D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr	2,600 ± 260	0.81	3,100 ± 720	0.83	1.21
Ac-Phe-D-Trp-Lys-Thr	51,500 ± 4,100	1.00	5,800 ± 1,000	0.80	0.11
Morphine·HCl	23 ± 2.4	1.20	27 ± 0.9	0.90	1.17

rosine for phenylalanine at position 3, which also resulted in an increase in μ-opiate receptor selectivity. Indeed, even the somewhat δ-selective [Pen^2, Cys^7]-octapeptide analog became μ-selective with the introduction of tyrosine at position 3.

The largest increase in receptor affinity resulted from the modification of the COOH-terminal carboxylic acid group. Both carboxamide analogs displayed high μ-opiate receptor affinity (Table 2). The tyrosine-containing analog was again slightly more potent with an ≈3-fold increase in μ-opiate receptor affinity, compared to the phenylalanyl analog. The tyrosine modification resulted in only a marginal increase in δ-opiate receptor affinity.

The binding studies also showed substantial differences in the way the two most potent analogs, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 and D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2, interacted with the radiolabeled ligands. In competition binding experiments against [^{3}H]naloxone, the Hill numbers were near unity, suggesting that these analogs were interacting with the receptor in a manner similar to that of the labeled antagonist. However, the Hill numbers obtained from binding experiments with [^{3}H]DADLE were substantially less than 1, indicating that the somatostatin octapeptide analogs were interacting with either multiple receptor sites or conformational states. For four analogs, the binding data from the studies using [^{3}H]DADLE were best fitted by computer analysis to a two-site model (Table 3), indicating high- and low-affinity sites with an approximately equal number of receptors in each group.

The ability of somatostatin and our conformationally restricted analogs to inhibit the binding of the biologically stable ^{125}I-labeled somatostatin analog CGP 23,996 to rat brain membranes was also examined (Table 4). Both somatostatin and unlabeled CGP 23,996 displayed high affinity for somatostatin receptors in the rat brain with IC_{50} values of 3.3 nM and 8.3 nM, respectively. The cyclic octapeptide compounds were substantially less potent, however. The most active analog, D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr, was less active by a factor of 50 than somatostatin in inhibiting the binding of ^{125}I-labeled CGP 23,996. The conformationally restricted analogs with the greatest μ-opiate receptor affinity displayed only weak activity at the somatostatin receptor. Indeed, the least potent compound at the opiate receptor, the [Pen^2, Cys^7]-octapeptide analog, was the second most active analog in inhibiting the binding of the labeled somatostatin derivative.

DISCUSSION

The somatostatin analog D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 binds to μ-opiate receptors in the rat brain with about 7800 times greater affinity than does the native hormone. The high μ-opiate receptor affinity of this analog, IC_{50} value 3.5 nM, is an order of magnitude greater than for any other somatostatin analog reported to date. Furthermore, this new analog and several others (Table 2) displayed high receptor selectivity. The IC_{50} (DADLE)/IC_{50} (naloxone) ratio of 271 is the largest ratio reported for any cyclic peptide; of the linear peptides, only morphiceptin displays greater μ-opiate receptor selectivity (16). In contrast to the linear peptide morphiceptin, however, the more conformationally restricted nature of the cyclic, penicillamine-containing somatostatin analogs should render them less subject to dynamic averaging. Careful conformational analysis, then, should provide important insights into the structural and conformational requirements for binding to the μ-opiate receptor.

In receptor binding studies using 10 nM [^{3}H]naloxone, a parallel shift in the binding curve of the analog D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 was obtained (data not shown). This suggests that, under the conditions of the assay, the cyclic octapeptide was acting at the μ-opiate receptor in a competitive manner and not allosterically (17). Competition binding experiments with the labeled antagonist nal-

Table 3. Inhibition of [^{3}H]DADLE binding to rat brain homogenates by somatostatin analogs fitted to a two-receptor-site model

Peptide	[^{3}H]DADLE			
	$IC_{50}(1)$	$IC_{50}(2)$	% $B_{max}(1)$	% $B_{max}(2)$
D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2	19	24,000	43	56
D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2	7	22,000	43	60
D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr	260	8,100	29	72
D-Phe-Pen-Tyr-D-Trp-Lys-Thr-Cys-Thr	640	51,000	51	46

B_{max}, concentration at which binding is maximal.

Table 4. Inhibition of ^{125}I-labeled CGP 23,996 binding to rat brain homogenates by somatostatin and its analogs

Peptide	IC_{50}, nM*
Somatostatin	3.3 ± 0.30
CGP 23,996	8.3 ± 2.0
D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr	170 ± 99
D-Phe-Pen-Phe-D-Trp-Lys-Thr-Cys-Thr	400 ± 200
D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2	690 ± 220
D-Phe-Pen-Phe-D-Trp-Lys-Thr-Pen-Thr	800 ± 300
D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr	980 ± 680
D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2	1500 ± 470
D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr	1600 ± 460
D-Phe-Pen-Tyr-D-Trp-Lys-Thr-Cys-Thr	4000 ± 780
Ac-Phe-D-Trp-Lys-Thr	7100 ± 870

*Inhibition of ^{125}I-labeled CGP 23,996.

oxone yielded Hill numbers near unity, indicating a similar binding mode to the μ-opiate receptor. This suggests that the somatostatin analogs may be acting as antagonists at the μ-opiate receptor, in agreement with previous reports of other somatostatin analogs (2–4).

While the structural and conformational features important for the opioid activity of the somatostatin analogs are not yet clear, the increase in receptor affinity that results from the substitution of tyrosine for phenylalanine at position 3 may be related to the well-known requirement for a phenolic hydroxyl moiety in the opiate and enkephalin systems. That the increase in potency is only 3-fold suggests, however, that a different mechanism may be involved. The nature of the COOH terminus is obviously important. The increase in μ-opiate receptor affinity that results from the transformation of the COOH-terminal threonine carboxylic acid group to a carboxamide is similar to that observed with the COOH-terminal alcohol analog D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol) (4). The increased potency of these peptides is probably not related to a general increase in lipophilicity, however. The octanol–water partition coefficients of the somatostatin peptides (Table 1) do not correlate well with receptor affinity; thus, other features, such as conformational changes, may be important.

The competition binding studies utilizing ^{125}I-labeled CGP 23,996 indicated that the selectivity of the cyclic octapeptide analogs extends to their interaction with the somatostatin receptor system. The most potent μ-opiate receptor analog, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2, was 1/200th as active as somatostatin in inhibiting the binding of the labeled CGP 23,996 analog. These studies show that there are substantial differences in the opiate and somatostatin receptor systems and in the structural and conformational features that are required for strong receptor interactions.

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