Characterization and Solubilization of Cyclo(His-Pro) Binding from Rat Liver Membranes[1] (42895)

MASATOMO MORI,[2] MASANOBU YAMADA, TOKUJI IRIUCHIJIMA, MASAMI MURAKAMI, AND SETSUO KOBAYASHI

First Department of Internal Medicine, Gunma University, School of Medicine, Maebashi 371, Japan

Abstract. **We have found cyclo(His-Pro) binding in rat liver plasma membranes. This study focused on the characterization of solubilized binding for cyclo(His-Pro) in rat liver membranes. The cyclo(His-Pro) binding of liver membranes was solubilized by digitonin and octyl-glucopyranoside. The efficiency of solubilization with digitonin was greater. However, cyclo(His-Pro) binding was not solubilized by Triton X-100, CHAPS, or Lubrol. Digitonin-solubilized membranes showed cyclo(His-Pro) binding with a high affinity constant (17 n*M*) and a low binding capacity (38 fmol/mg protein). Lectins from wheat germ, *Bandeiraea simplicifolia II*, *Dolichos biflorus*, Glycine max, and *Tetragonolobus purpureas* significantly adsorbed [^{3}H]cyclo(His-Pro)-binding complex, but *Bandeiraea simplicifolia I*, *Ricinus communis I*, or *Lens culinaris* did not adsorb the binding complex. An analysis of [^{3}H]cyclo(His-Pro)-associated membranes by high performance gel filtration chromatography showed a radioactive peak of *M*$_r$ 200,000. These data indicate that cyclo(His-Pro) binding of rat liver membranes is solubilized by digitonin and is a glycoprotein of *M*$_r$ 200,000.** [P.S.E.B.M. 1989, Vol 191]

Histidyl-proline diketopiperazine [cyclo(His-Pro)] is one member of the neuropeptide family which was first characterized as a degradation product of thyrotropin-releasing hormone (TRH) in the rat brain (1). The fact that cyclo(His-Pro) is distributed throughout the brain (2, 3) and possesses varying neurologically active functions (4, 5) suggests that cyclo(His-Pro) may function as a neuromodulator in the brain. Because the action of neuropeptides is initiated by their binding to the plasma membranes of cells in the brain, attempts have been made to identify and distinguish cyclo(His-Pro) binding of brain cell membranes (6–9). Surprisingly, little information is as yet available in regard to the binding nature of this important regulatory neuropeptide in the brain.

Detection of cyclo(His-Pro) immunoreactivity in a wide variety of tissues outside the brain (10) and of many biologic actions irrelevant to the brain function has led to the proposal that cyclo(His-Pro) has broad functions in addition to its role as a brain neuromodulator. Such actions include suppression of cholesterol synthesis in the gastrointestinal tract (11) and inhibition of insulin secretion (12). Recently, we found that significant binding of cyclo(His-Pro) to rat liver plasma membranes occurred. This binding was highly specific for cyclo(His-Pro) with a high affinity constant and a low binding capacity. The binding was decreased by repeated administration of cyclo(His-Pro). The data suggest that cyclo(His-Pro) binding to liver membranes may be receptor in nature (9). This distinctive binding of liver membranes for cyclo(His-Pro) has been reported to be associated with testosterone (13). Because advanced insight into the nature of receptors and the mechanisms of many neuropeptides has been obtained by structural characterization of receptors (14–16), solubilization and characterization of cyclo(His-Pro) binding would provide both valuable information and a useful tool for further studies. In this study we report the first characterization of solubilized cyclo(His-Pro) binding from rat liver membranes.

Materials and Methods

Preparation of Plasma Membranes. As described previously (9), the crude plasma membranes were obtained from livers of 4- to 5-week-old male rats of the

[1] Presented in part at the 8th International Congress of Endocrinology, Kyoto, Japan, 1988 (Abstract 04-18-008).

[2] To whom all correspondence and requests for reprints should be addressed.

Received September 1, 1988. [P.S.E.B.M. 1989, Vol 191]
Accepted January 25, 1989.

0037-9727/89/1912-0108$02.00/0

Wistar strain. Animals were anesthetized with ether and trunk blood drawn through the abdominal aorta. The livers were rinsed with saline, weighed, homogenized in 10 volumes of TKMN (40 m*M* Tris-HCl, 2 m*M* KCl, 2 m*M* $MgCl_2$, 7 m*M* NaCl, pH 7.4) buffer containing 250 m*M* sucrose, and centrifuged at 1000*g* for 10 min. The supernatant was further centrifuged at 53,000*g* for 30 min. The resulting supernatants were discarded and the precipitates saved.

Solubilization of Membranes. The plasma membranes were resuspended in 40 m*M* Tris-HCl (pH 7.4) buffer containing varying doses of detergents, incubated at 0°C for 30 min, and then centrifuged at 100,000*g* for 60 min. The supernatants were used for cyclo(His-Pro) binding. Detergents used were digitonin, Triton X-100, CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate), Lubrol, and *n*-octyl-β-D-glucopyranoside (all purchased from Sigma Chemical Co., St. Louis, MO).

Binding Assay. Cyclo(His-Pro) binding was estimated according to the basis of our previous data which showed the saturable and specific binding of cyclo(His-Pro) for liver membranes (9). Two-hundred microliters of freshly solubilized membranes were incubated with 10 μl of 1 pmol of [^{3}H]cyclo(His-Pro) (specific activity = 38.4 Ci/mmol; New England Nuclear, Boston, MA) at 0°C for 120 min. As nonspecific binding, 2 nmol of unlabeled cyclo(His-Pro) (Tanabe Pharmaceutical Co., Osaka, Japan) in 10 μl of Tris-HCl buffer were added. As described by Penefsky (17), a rapid gel filtration technique was used to separate the bound form from the free form of cyclo(His-Pro). Incubation was terminated by layering 100 μl of the reaction mixture over a Sephadex G-50 minicolumn, in which the gel was packed in a 1-ml plastic tuberculin syringe, preequilibrated with 40 m*M* Tris-HCl buffer, and previously centrifuged at 100*g* for 2 min. The loaded minicolumn was then centrifuged at 100*g* for 2 min, and 100 μl of Tris-HCl buffer were layered on a column followed by centrifugation at 100*g* for 2 min. The eluents were collected in scintillation vials. The eluents from the Sephadex minicolumn recovered over 90% of solubilized protein (the eluent, 0.59 vs the original protein, 0.64 mg, $n = 2$) and less than 1% of free [^{3}H]cyclo(His-Pro) (the eluent, 1.1×10^3 vs the original radioactivity, 125×10^3 dpm, $n = 2$). This suitable condition was not obtained by using Sephadex G-10, G-25, G-100, or G-200 gel. Four milliliters of Aquasol-2 (New England Nuclear) were added to vials, and the concentration of [^{3}H]cyclo(His-Pro) receptor complex was determined by counting the radioactivity of the collected eluent. Specific binding was defined by subtracting the nonspecific binding (with unlabeled cyclo(His-Pro)) from the total binding (without unlabeled dipeptide). The binding ratio (%) of [^{3}H]cyclo(His-Pro) added to the solubilized membranes was 2.15 and 1.04 as the total and nonspecific binding, respectively.

Double Reciprocal Plot of Specific Bound. The values of dissociation constant and maximum binding capacity of cyclo(His-Pro) binding were determined according to the method of Akera and Cheng (18) using a double reciprocal plot. The solubilized membranes were incubated with varying doses of [^{3}H]cyclo(His-Pro) in the presence or absence of a 1000-fold concentration of unlabeled cyclo(His-Pro), and the specific binding of cyclo(His-Pro) was determined as described above. Because the specific binding progressively elevated with the increasing doses (0.2–6 pmol) of [^{3}H]cyclo(His-Pro) and the binding with 6- to 9-pmol doses of [^{3}H]cyclo(His-Pro) reached the plateau, the doses ranging from 0.2 to 9 pmol of [^{3}H]cyclo(His-Pro) were used to estimate a double reciprocal plot.

Lectin Adsorption. Because 20 m*M* Hepes buffer (pH 7.4) could substitute for 20 m*M* Tris-HCl buffer (pH 7.4) in labeling liver membranes with [^{3}H]cyclo(His-Pro), and 20 m*M* NaH_2PO_4 buffer (pH 7.4) reduced [^{3}H]cyclo(His-Pro) binding by 60%, liver membranes were suspended in 20 m*M* Hepes buffer containing 1.0% digitonin. The membranes were incubated at 0°C for 30 min and centrifuged at 100,000*g* for 60 min. The supernatant was incubated with 4 pmol of [^{3}H]cyclo(His-Pro) at 0°C for 120 min, and eluents of [^{3}H]cyclo(His-Pro)-binding complex were obtained as described above. They were used to determine the glycoprotein nature using various lectins which were coupled with agarose. Lectins used were wheat germ agglutinin, *B. simplicifolia I* (Vector Laboratories, Inc., Burlingame, CA). *D. biflorus*, glycine max, *T. purpureas*, *B. simplicifolia II*, *R. communis I*, and *Lens culinaris* (Sigma Chemical Co.). Each lectin-agarose was previously washed with 20 m*M* Hepes buffer containing 0.1% digitonin. One-hundred microliters of [^{3}H]cyclo(His-Pro)-binding complex were then incubated with 0.1 mg of each lectin-agarose in 0.1 ml of Hepes buffer containing 0.1% digitonin. After incubation at 0°C for 3 hr, the incubation mixture was centrifuged at 1500*g* for 20 min, and 100 μl of each supernatant were transferred into a scintillation vial for counting ^{3}H. The efficiency of adsorption was calculated by subtracting the supernatant ^{3}H count from the activity of the original [^{3}H]cyclo(His-Pro)-binding complex.

High Performance Gel Filtration Chromatography (HPGFC) Analysis. Digitonin-solubilized membrane extract was labeled with [^{3}H]cyclo(His-Pro) and [^{3}H]cyclo(His-Pro)-binding complex was obtained as described above. This binding complex and ^{14}C-labeled protein markers were applied on an HPGFC column (Zorbax GF-250, 9.4 × 250 mm; Du Pont Co., Wilmington, DE) and eluted with 0.2 *M* K_2HPO_4 (pH 7.4) at a speed of 1.0 ml/min as described elsewhere (19). Collected samples were used for counting of ^{3}H and ^{14}C. Protein markers used were [^{14}C]ovalbumin, [^{14}C]albumin, [^{14}C]immunoglobulin G, and [^{14}C]myosin

(New England Nuclear; M_r = 46,000, 69,000, 150,000, and 200,000 respectively).

Protein concentration was determined according to the method of Smith *et al.* (20) using bicinchoninic acid (Pierce Chemical Co., Rockford, IL).

Results

Figure 1 shows the efficiency of detergents in solubilizing cyclo(His-Pro) binding from liver membranes. One percent of digitonin and octyl-glucopyranoside solubilized, respectively, 36% and 23% of the original cyclo(His-Pro) binding, but neither Lubrol, CHAPS, nor Triton X-100 significantly solubilized cyclo(His-Pro) binding from liver membranes. The detailed concentrations (0.125–2%) of digitonin and octyl-glucopyranoside to solubilize liver cyclo(His-Pro) binding were determined (data not shown here). One and 2% of digitonin solubilized, respectively, 35% and 39.6% of the original cyclo(His-Pro) binding, and the same concentrations of octyl-glucopyranoside solubilized cyclo(His-Pro) binding from liver membranes by 25.4% and 28.6%, respectively. Therefore, 1–2% of digitonin was used to solubilize cyclo(His-Pro) binding from liver membranes. [^{3}H]Cyclo(His-Pro) binding was found to show the time- and protein concentration-dependent increase in 1% digitonin-solubilized liver membranes (Fig. 2).

Figure 3 shows a double reciprocal plot of the specific cyclo(His-Pro) binding data. A straight line was observed. The dissociation constant and maximal binding were estimated to be 17.0 ± 4.0 nM (n = 3) and 38 fmol/mg protein, respectively. The [^{3}H]cyclo(His-Pro) binding was not significantly decreased by TRH, LHRH, or somatostatin in doses of 1 μM (data not shown here). The data are compatible with the previous results showing the highly specific binding of cyclo(His-Pro) for liver membranes (9).

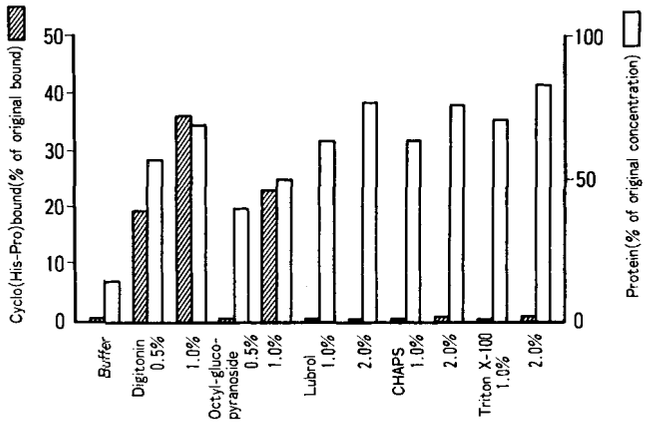

Figure 1. Solubilization of [^{3}H]cyclo(His-Pro)-binding complex by detergents. Liver plasma membranes were solubilized by detergents and used for determination of binding for [^{3}H]cyclo(His-Pro) as described in Materials and Methods. Values are the mean of duplicate determinations.

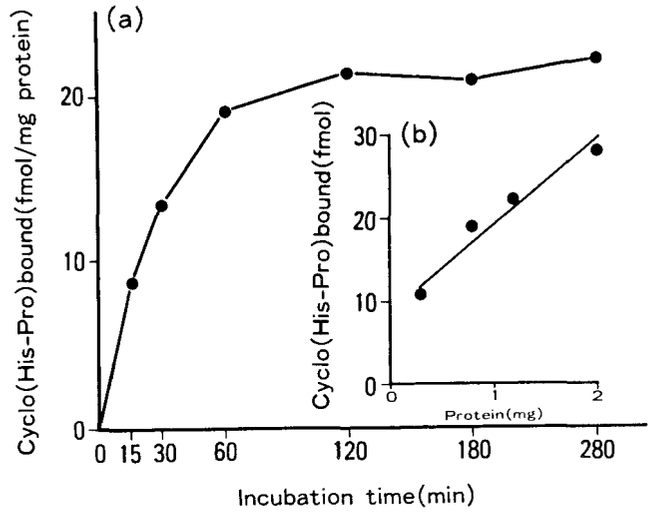

Figure 2. Time- and protein concentration-dependent binding of [^{3}H]cyclo(His-Pro) in solubilized liver membranes. Liver membranes solubilized by digitonin were incubated with [^{3}H]cyclo(His-Pro) at 0°C. After incubation, specific binding was determined as described in Materials and Methods. Values are mean of duplicate determination. (a) Time course of specific [^{3}H]cyclo(His-Pro) binding. (b) Effects of membrane concentration on [^{3}H]cyclo(His-Pro) binding.

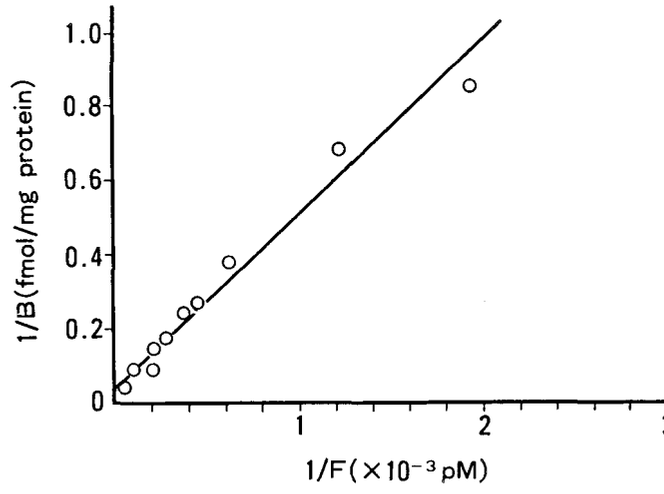

Figure 3. A double reciprocal plot of the specific cyclo(His-Pro) binding data. Solubilized liver membranes were incubated with increasing doses of [^{3}H]cyclo(His-Pro) and cyclo(His-Pro)-binding complex was determined using a rapid gel filtration technique as described in Materials and Methods. B, the amount of cyclo(His-Pro) specifically bound; F, the amount of free cyclo(His-Pro).

Lectins from wheat germ agglutinin, *B. simplicifolia II* (both related with *N*-acetyl-D-glucosamine), *D. biflorus*, glycine max (both related with *N*-acetyl-D-galactosamine), and Tetragonolobus purpureas (related with α-fucosyl) significantly precipitated [^{3}H]cyclo(His-Pro)-binding complex (Table I). However, *B. simplicifolia I*, *R. communis* (both related with D-galactosyl), or *L. culinaris* (related with D-mannosyl) did not adsorb the binding complex.

Figure 4 shows an analysis of [^{3}H]cyclo(His-Pro) binding complex by HPGFC. The radioactive peak of

Table I. Adsorption of the [^{3}H]Cyclo(His-Pro)-Binding Complex by Lectins[a]

Lectins	% adsorption of the original binding complex activity
Wheat germ	28.4 ± 2.9
B. simplicifolia II	35.0 ± 6.3
D. biflorus	36.4 ± 5.6
Glycine max	25.4 ± 8.6
T. purpureas	46.0 ± 11.2
B. simplicifolia I	5.6 ± 2.8
R. communis I	8.1 ± 4.9
L. culinaris	5.8 ± 4.2

[a] As described in Materials and Methods, [^{3}H]cyclo(His-Pro)-binding complex was incubated with 0.1 mg of each lectin at 0°C for 3 hr. Incubation mixture was then centrifuged, and the supernatant was counted. The efficiency of adsorption of lectin was calculated by subtracting the supernatant ^{3}H count from radioactivity of the original [^{3}H]cyclo(His-Pro)-binding complex. Three samples were used in each group. The data are expressed as mean ± SEM.

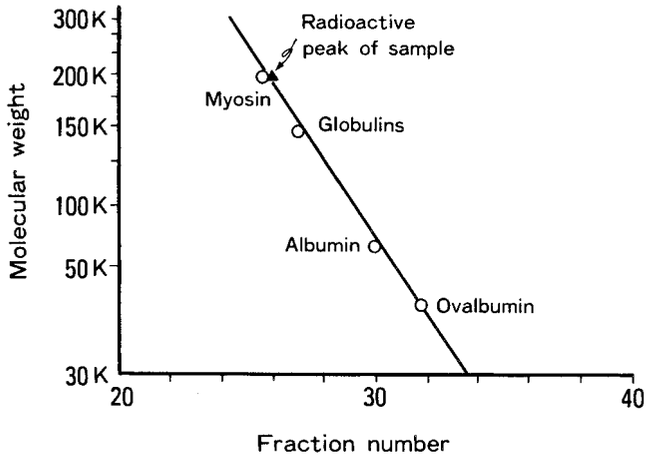

Figure 4. An analysis of [^{3}H]cyclo(His-Pro)-receptor complex by HPGFC. [^{3}H]Cyclo(His-Pro)-binding complex and ^{14}C-labeled protein markers were applied on an HPGFC column (Zorbax GF-250, 9.4 × 250 mm) and eluted with 0.2 *M* K_2HPO_4 (pH 7.4) at a speed of 1.0 ml/min. Collected samples were used for counting of ^{3}H and ^{14}C.

binding complex corresponded to a marker of [^{14}C] myosin, indicating that the M_r of [^{3}H]cyclo(His-Pro) binding was approximately 200,000 which may include digitonin M_r (=1229).

Discussion

This study describes the development of a protocol suitable for obtaining binding sites of liver membranes for [^{3}H]cyclo(His-Pro) in a soluble form. The first critical step in our studies was to determine how to separate the [^{3}H]cyclo(His-Pro) bound form from free dipeptide. Although several methods such as the dextran-coated charcoal (21), polyethylene glycolfiltration (22), equilibrium dialysis (23), and DEAE separation methods (22) were used to detect peptide- and hormone-binding receptor complex, all of them were not appropriate for detection of [^{3}H]cyclo(His-Pro) associated with macromolecular components of solubilized liver membranes (data not shown here). Solubilized binding activity was reproducibly and conveniently detected by using a rapid gel filtration technique (17).

Because the previous data showed that cyclo(His-Pro) binding was lost with increasing temperature (9), in the series of present studies, we used the cooling conditions to minimize the possible degradation of solubilized cyclo(His-Pro) receptor. However, the half-life of the solubilized sites remains to be seen.

Solubilization of cyclo(His-Pro) binding is necessary for detailed structural characterization of the protein. Triton X-100 solubilized the TRH receptor from rat brains (24) and the TSH receptor from adipocytes (25), CHAPS solubilized the LHRH receptor from pituitary membranes (15) and the parathyroid hormone receptor from renal membranes (21), and ocytl-glucopyranoside solubilized the vasoactive intestinal peptide receptor from lung membranes (26). Indeed, the present study showed that 1–2% Triton X-100, CHAPS, and Lubrol could solubilize more than 60% of the protein from liver membranes. However, these solubilized proteins did not show the significant binding for [^{3}H] cyclo(His-Pro). The approach in the present study to solubilization of cyclo(His-Pro) receptor from liver membranes used digitonin which was most effective among nondenaturing detergents tested. Under the conditions in which 1% digitonin was used, approximately 35% of the cyclo(His-Pro) binding sites were extracted.

Significant and saturable binding of [^{3}H]cyclo(His-Pro) to digitonin-solubilized liver membranes was observed. By means of an analysis of double reciprocal plot, the K_d value of [^{3}H]cyclo(His-Pro) to liver membranes was calculated to be 17 (10–24) n*M* which was slightly smaller than that obtained for binding in intact liver membranes (9). This difference has definitely not been explained. The possibility was raised that the concentrations of [^{3}H]cyclo(His-Pro) (0.2–9 pmol) used in this study differed from those (10–40 pmol) in the previous study. Consequently, the binding curves were shifted to lower cyclo(His-Pro) concentrations, yielding a small value of K_d compared with its value as determined in the previous study. Alternatively, the method of a double reciprocal plot analysis to estimate K_d may give a different value, although the previous data showed that the K_d value of liver cyclo(His-Pro) membranes determined using a double reciprocal plot analysis was similar to that using a Scatchard plot analysis (9). It remains to be seen whether digitonin used in the present study affected the constant for dissociation of [^{3}H]cyclo(His-Pro) binding.

The present observations that solubilized cyclo(His-Pro)-binding complex interacted with various lectins have provided information with respect to the carbohydrate nature of the protein. Cyclo(His-Pro) binding was found to interact to a substantial extent with wheat germ, *B. simplicifolia II*, *D. biflorus*, Glycine

max, and *T. purpureas*, but not significantly with *B. simplicifolia I*, *R. communis I*, or *L. culinaris*. Therefore, the cyclo(His-Pro)-binding complex is a glycoprotein containing *N*-acetyl-D-glucosamine and galactosamine and also terminal fucosyl residues. The affinity of lectin for the solubilized bindings would provide a method for partial purification of the cyclo(His-Pro) binding complex, an important step for more detailed characterization.

An HPGFC analysis of cyclo(His-Pro)-binding complex revealed a protein peak of M_r 200,000. Little information is available concerning the extent to which digitonin may affect molecular size of cyclo(His-Pro) binding in liver membranes. The M_r of liver cyclo(His-Pro) binding was comparable to those of brain TRH receptor (300,000) (24), pancreatic somatostatin receptor (200,000) (27), and renal parathyroid hormone receptor (180,000) (21), but was significantly higher than those of thyroid TSH receptor (50,000) (25), pituitary LHRH receptor (60,000) (28), and pituitary somatostatin receptor (88,000) (16). Further investigations are required before a clearly defined structural model of cyclo(His-Pro) binding complex can be presented.

1. Prasad C, Peterkofsky A. Demonstration of two separate enzymatic activities for the degradation of thyrotropin-releasing hormone in hamster hypothalamic extracts. J Biol Chem **251**:3229–3234, 1976.
2. Mori M, Prasad C, Wilber JF. Regional dissociation of histidyl-proline diketopiperazine and thyrotropin-releasing hormone in the rat brain. Brain Res **231**:451–453, 1982.
3. Mori M, Jayaraman A, Prasad C, Pegues J, Wilber JF. Distribution of histidyl-proline diketopiperazine (cyclo(His-Pro)) and thyrotropin-releasing hormone (TRH) in the primate central nervous system. Brain Res **245**:183–186, 1982.
4. Prasad C, Mori M, Wilber JF, Pierson W, Pegues J, Jayaraman A. Distribution and metabolism of cyclo(His-Pro): A new member of the neuropeptide family. Peptides **3**:591–598, 1982.
5. Peterkofsky A, Battaini F, Koch Y, Takahara Y, Dannies P. Histidyl-proline diketopiperazine: Its biological role as a regulatory peptide. Mol Cell Biochem **42**:45–63, 1982.
6. Koch Y, Battaini F, Peterkofsky A. [^{3}H]Cyclo(His-Pro) in rat tissues: Distribution, clearance and binding. Biochem Biophys Res Commun **104**:823–829, 1982.
7. Bataini F, Koch Y, Takahara Y, Peterkofsky A. Specific binding to adrenal particulate fraction of cyclo(Histidyl-Proline), a TRH metabolite. Peptides **4**:89–96, 1983.
8. Prasad C, Edwards RM. Down regulation of adrenocortical cyclo(His-Pro)-binding sites by cyclo(His-Pro) administration to neonatal but not adult rats. Neurosci Lett **49**:165–169, 1984.
9. Mori M, Yamada M, Yamaguchi M, Suzuki M, Ohshima K, Kobayashi I, Kobayashi S. Cyclo(His-Pro), a metabolite of thyrotropin-releasing hormone: Specific binding to rat liver membranes. Biochem Biophys Res Commun **134**:443–451, 1986.
10. Mori M, Pegues J, Prasad C, Edwards R, Wilber JF. Distribution and characterization of cyclo(His-Pro)-like immunoreactivity in the rat gastrointestinal tract. Biochem Biophys Res Commun **109**:982–987, 1982.
11. Gebhard RL, Morley KE, Prigge WF, Goodman MW, Prasad C. TRH and histidyl-proline diketopiperazine inhibit cholesterol synthesis in dog intestine. Peptides **2**:137–140, 1981.
12. Wilber JF, Mori M, Kandarakis ED Iriuchijima T, Sacks H, Kitabachi AE. Histidyl-proline diketopiperazine [cyclo(His-Pro)]: A new neuropeptide modulator of insulin and glucagon secretion. Trans Assoc Am Physicians **97**:88–94, 1984.
13. Sora YH, Ogawa N, Mori A. Changes in rat liver cyclo(His-Pro) binding sites upon castration and testosterone administration. Regul Peptides **18**:1–5, 1987.
14. Hinkle PM, Lewis DG. Solubilization of pituitary receptors for thyrotropin-releasing hormone. Biochim Biophys Acta **541**:347–359, 1978.
15. Perrin MH, Haas Y, Rivier JE, Vale WW. Solubilization of the gonadotropin-releasing hormone receptor from bovine pituitary plasma membranes. Endocrinology **112**:1538–1540, 1983.
16. Lewis LD, Williams JA. Structural characterization of the somatostatin receptor in rat anterior pituitary membranes. Endocrinology **121**:486–492, 1987.
17. Penefsky HS. Reversible binding of P_i by beef heart mitochondrial adenosine triphosphatase. J Biol Chem **252**:2891–2899, 1977.
18. Akera T, Cheng V-JK. A simple method for the determination of affinity and binding site concentration in receptor binding studies. Biochim Biophys Acta **470**:412–423, 1977.
19. Sivakoff SI. HPLC analysis and purification of antibody conjugates. Biochromatography **1**:42–48, 1986.
20. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto ER, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem **150**:76–85, 1985.
21. Nissenson RA, Mann E, Winer J, Teitelbaum AP, Arnaud CD. Solubilization of a guanine nucleotide-sensitive parathyroid hormone-receptor complex from canine renal cortex. Endocrinology **118**:932–939, 1986.
22. Cremo CR, Herron GS, Schimerlik MI. Solubilization of the atrial muscarinic acethylcholine receptor: A new detergent system and rapid assays. Anal Biochem **115**:331–338, 1981.
23. Catterall WA, Morrow CS, Hartshorne RP. Neurotoxin binding to receptor sites associated with voltage-sensitive sodium channels in intact, lysed, and detergent-solubilized brain membranes. J Biol Chem **254**:11379–11387, 1979.
24. Ogawa N, Yamawaki Y, Kuroda H, Nukina I, Ota Z, Fujino M, Yanaihara N. Characteristics of thyrotropin-releasing hormone (TRH) receptors in rat brain. Peptides **3**:669–677, 1982.
25. Iida Y, Amir SM, Ingbar SH. Stabilization, partial purification, and characterization of thyrotropin receptors in solubilized guinea pig fat cell membranes. Endocrinology **121**:1627–1636, 1987.
26. Provow S, Velicelebi G. Characterization and solubilization of vasoactive intestinal peptide receptors from rat lung membranes. Endocrinology **120**:2442–2452, 1987.
27. Strikant CB, Patel YC. Somatostatin receptors on rat pancreatic acinar cells. J Biol Chem **261**:7690–7696, 1986.
28. Eidne KA, Hendricks DT, Millar RP. Demonstration of a 60K molecular weight luteinizing hormone-releasing hormone receptor in solubilized adrenal membranes by a ligand-immunoblotting technique. Endocrinology **116**:1792–1795, 1985.