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CYCLO(HIS-PRO), A METABOLITE OF THYROTROPIN-RELEASING HORMONE: SPECIFIC BINDING TO RAT LIVER MEMBRANES

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Summary: [^{3}H]cyclo(His-Pro) bound with high affinity (59 nM) to a single class of sites in rat liver plasma membranes, without significant tracer degradation during equilibration for 60 min at 0°C. Binding was specific and saturable (3.9 pmol/mg protein), and were increased by the addition of K^{+}, Mg^{++} and Na^{+} at optimal concentrations, but not of Ca^{++} at all concentrations tested. In vivo administration of cyclo(His-Pro), but not thyrotropin-releasing hormone, to rats caused the downregulation of cyclo(His-Pro)-binding sites with decreases in specific binding numbers but did not change binding affinity.

Histidyl-proline diketopiperazine or cyclo(His-Pro), a metabolite of thyrotropin-releasing hormone (TRH) by pyroglutamate aminopeptidase (1), is present in the brain (2,3), pancreas (4), gastrointestinal tract (5) and peripheral blood (6). This dipeptide has been proposed as a bioactive substance showing many biological activities (7,8). However, the detail mechanism(s) by which cyclo(His-Pro) produces these activities remains unknown. Although bioactive effects of neuropeptides and hormones might occur through their specific receptor binding sites in target tissues (9), there is no report concerning the specific binding sites of cyclo(His-Pro) in any tissues except for the adrenal gland, in which a binding affinity and capacity of cyclo(His-Pro) were observed to be respectively low and high (10,11). The physiological significance of this low affinity binding sites, however, is obscure, because administration of dipeptide did not cause any changes in cyclic nucleotide

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formation in the adrenal gland (10). The previous study demonstrated the obvious uptake of tritium-labeled cyclo(His-Pro) by the rat liver as well as the adrenal gland (12). In the liver tissue the binding site of cyclo(His-Pro) has not been yet characterized . Here we show specific binding of dipeptide in the rat liver plasma membranes with high affinity.

MATERIALS AND METHODS

1. Peptides: Cyclo(His-Pro), TRH, acid TRH and Glu-His-ProNH$_2$ were supplied by Tanabe Pharmaceutical Co., Osaka, Japan. pGlu-His-Gly (anorexigenic peptide), luteinizing hormone-releasing hormone (LHRH) and growth hormone-release inhibiting factor (GHIF) were purchased from Peninsula Laboratories, Inc., Belmont CA. Histidine, proline and pyroglutamate were purchased from Sigma Chemical Co., St. Louis, MO. [^{3}H-Pro]cyclo(His-Pro) was purchased from New England Nuclear, Boston, MA (specific activity = 97Ci/mmol).

2. Preparation of membranes: The crude plasma membranes of the rat liver were obtained according to the method of Burt and Snyder (13). Adult male Wistar rats weighing 200-300g were anaesthetised and trunk blood was drawn through the abdominal aorta. The liver was rinsed with saline, weighed, homogenized in 10 vol of 0.32 M sucrose using a motor-drive pestle (20 strokes) and centrifuged at 1,000xg for 10 min. The supernatant was further centrifuged at 24,000xg for 40 min. The resulting precipitate was resuspended in TKMN buffer (40 mM Tris-HCl, 2 mM KCl, 2 mM $MgCl_2$, 7 mM NaCl, pH 7.4) containing 0.1% bovine serum albumin (BSA). The membrane protein concentration was determined by the method of Lowry et al.

3. Binding assay: Two hundreds µl of freshly prepared homogenate (approximately 0.5 mg protein) was incubated with 10 µl of 1.8 pmol [^{3}H]cyclo(His-Pro) in the presence or absence of 2 nmol unlabeled cyclo(His-Pro) at 0°C for 60 min. The total volume was 220 µl and each assay was performed in duplicate. After addition of 0.5 ml of chilled TKMN buffer to terminate the incubation, membrane-bound [^{3}H]cyclo(His-Pro) was separated from free peptide by filtration under reduced pressure through GF/B filters (Whatman, 2.5 cm). The filters were washed with 5 ml of chilled TKMN buffer, dryed on a heating plate and assayed for radioactivity with 5 ml of scintillation fluid (Econofluor from New England Nuclear) in a Sigma scintillation spectrometer. Specific bound of cyclo(His-Pro) was calculated by subtracting a non-specific counting in the presence of unlabeled peptide from a total counting in the absence of peptide.

4. Chromatographic characterization of bound cyclo(His-Pro): As described above, [^{3}H]cyclo(His-Pro) was incubated with liver plasma membrane at 0°C for 60 min and bound cyclo(His-Pro) was trapped by filters. Membrane bound radioactivity on filters was extracted with 5 ml of 99% methanol. The methanol extracts of five samples were pooled, evaporated to dryness and dissolved in 100 µl of water. Samples of membrane bound radioactivity, standard [^{3}H]cyclo(His-Pro) and authentic cyclo(His-Pro) were separately applied to a silica gel-60 TLC plate. The

chromatogram was developed in a solvent system of $CHCl_3$-CH_3OH-27%NH_4OH (7:3:1) in an ascending mode. After drying, the plates were cut into 0.5 cm-wide strips, extracted with 1 ml 99% methanol and counted for radioactivity with 4 ml scintillation fluid. A marker of authentic cyclo(His-Pro) was identified by spraying with the Pauly reagent. The Rf values of proline-containing peptides in this system were as follows: cyclo(His-Pro), 0.78;His-Pro, 0.22;Pro-NH_2, 0.62;Pro, 0.10;TRH, 0.50;acid TRH, 0.24.

5. In vivo administration of cyclo(His-Pro) and TRH: Adult male rats were daily injected intraperitoneally with 0.5 mg/100g body weight of cyclo(His-Pro) or TRH (b.i.d.) for 3 days. Animals were killed 6 h after the last injection, and cyclo(His-Pro)-binding sites in liver membranes were assayed as described above.

RESULTS

Binding of [^{3}H]cyclo(His-Pro) to Membranes. Fig. 1 depicts that specific binding of cyclo(His-Pro) to liver membranes was dependent on period of incubation, protein concentration of membranes and temperature. At 0°C and pH 7.4 specific binding of cyclo(His-Pro) increased progressively with incubation time, reaching a maximum at 60 min (Fig. 1a), and was linear function of membrane concentrations, which varied from 0.1 to 1.0 mg protein per tube (Fig. 1b). Specific binding at pH 7.4

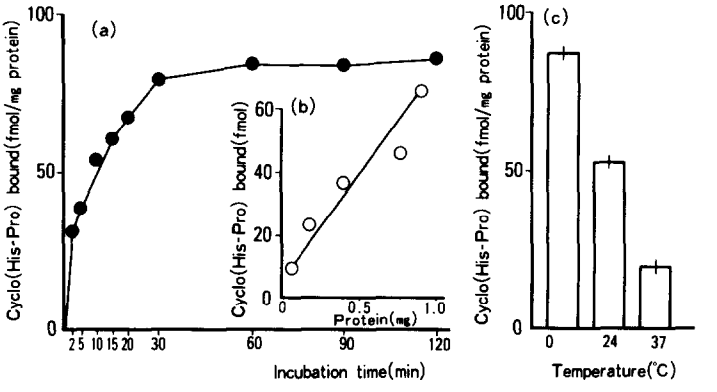


Fig. 1. A. Time course of [^{3}H]cyclo(His-Pro) association. As described in Materials and Methods, liver plasma membranes (about 0.5 mg protein) were incubated with TKMN buffer containing [^{3}H]cyclo(His-Pro) at 0°C. Determination of specific binding is described in Methods. Values are means of triplicate determinations. B. Effects of amount of membrane protein on the specific binding of [^{3}H]cyclo(His-Pro). C. Effect of incubation temperature on [^{3}H]cyclo(His-Pro) binding. Membrane protein (about 0.5 mg) was incubated at various temperature at 0°C for 60 min and the specific binding calculated as described in Methods.

Table 1. Effects of salt on specific binding of [^{3}H]cyclo(His-Pro)

Cation	Concentration (mM)	Specific binding*
KCl	2	119.2
	7	103.6
	20	90.2
	50	80.7
	100	81.3
$MgCl_2$	2	102.0
	7	96.9
	20	63.8
	50	38.9
	100	37.8
NaCl	2	84.0
	7	110.4
	20	110.1
	50	97.4
	100	107.3
$CaCl_2$	7	63.3
	20	50.1
	50	26.2
	100	11.8
KCl + $MgCl_2$	2 + 2	109.3
KCl + $NaCl^2$	2 + 7	113.3
KCl + NaCl	2 + 20	104.4
KCl + $MgCl_2$ + NaCl	2 + 2 + 7	135.9
KCl + $MgCl_2$ + NaCl	2 + 2 + 20	122.7

*Each value represents the percentage of bound cyclo(His-Pro) compared to the absence of salt. The standard assay buffer contained 40 mM Tris-HCl, pH 7.4 and 0.1% BSA. Binding of [^{3}H]cyclo(His-Pro) to liver membranes (approximately 0.5 mg protein) was performed as described in Methods.

exhibited a sharp temperature dependence (Fig. 1c). At 0°C the specific binding was influenced by pH (pH 5.5=42, pH 6.5=72, pH 8.4=85% of the specific binding at pH 7.4). Table 1 shows effects of various salts. There were significant increases in cyclo(His-Pro) binding after the addition of K^+ (2 and 7 mM), Mg^{++} (2 mM) and Na^+ (7 and 20 mM), with subsequent decreases at higher concentrations. The addition of Ca^{++} inhibited the specific binding at all concentrations. When the binding assay was performed in the presence of 2 mM K^+, 2 mM Mg^{++} and 7 mM Na^+, the specific binding increased to 135.9% of control in the absence of salt.

Equilibrium Binding Studies. Fig.2 (Left) shows that specific and non-specific bindings were linear functions of peptide concentrations. Fig. 2 (Right) shows a double reciprocal replot

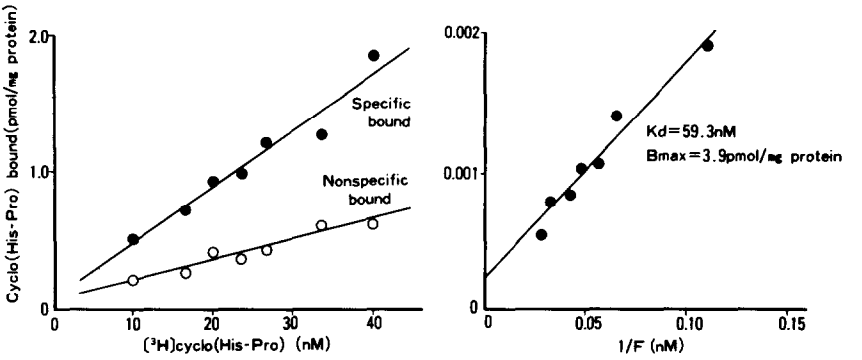


Fig. 2. Binding of [^{3}H]cyclo(His-Pro) as a function of radiolabeld peptide concentration (Left). Increasing amounts of [^{3}H]cyclo(His-Pro) were incubated with membrane preparation (0.5 mg protein) at 0°C for 60 min. Nonspecific and specific binding was determined as described in Methods. Double reciprocal replot of the amount of cyclo(His-Pro) specifically bound (B) versus the amount of free cyclo(His-Pro) (F) (Right).

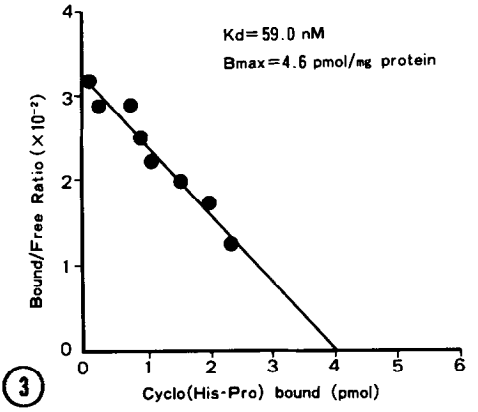
of the specific binding data yielding a straight line. On the basis of the previous experiment (14) the dissociation constant (Kd=59 nM) and maximal binding (Bmax=3.9 pmol/mg protein) were calculated from the present data.

Specificity of cyclo(His-Pro) Binding. Table 2 shows the ability of varying analogues of cyclo(His-Pro) to inhibit the specific cyclo(His-Pro) binding. Unlabeled cyclo(His-Pro) inhibited [^{3}H]cyclo(His-Pro) binding in a competitive manner and 0.1 μM cyclo(His-Pro) was required to inhibit [^{3}H]cyclo(His-Pro) binding by 50%. Scatchard plot analysis (15) from this

Table 2. Competition for Binding of [^{3}H]cyclo(His-Pro) by various compounds

Compound	IC50 ($Mx10^{-6}$)*
cyclo(His-Pro)	0.1
pGlu-His-ProNH$_2$ (TRH)	338
pGlu-His-ProOH (acid TRH)	2181
pGlu-His-Gly (anorexigenic peptide)	2000
Glu-His-ProNH$_2$	290
LHRH	453
GHIF	291
His	545
Pro	>4500
pGlu	3750

Binding of [^{3}H]cyclo(His-Pro) to liver membranes was performed as described in Methods. Each assay tube contained a crude plasma membrane corresponding to approximately 0.5 mg protein.
*The concentration required to inhibit cyclo(His-Pro) binding by 50%.



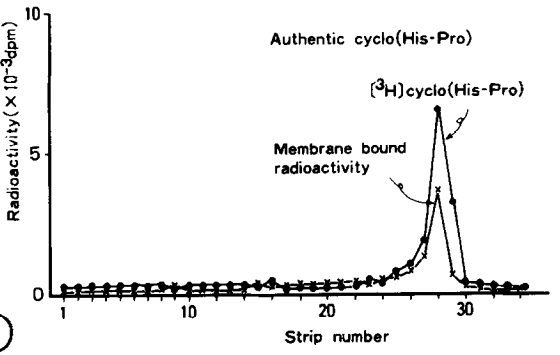


Fig. 3. Scatchard plot analysis of the data obtained from competitive inhibition of [^{3}H]cyclo(His-Pro) binding by unlabeled cyclo(His-Pro). Liver membranes were incubated with TKMN buffer containing [^{3}H]cyclo(His-Pro) in the presence of various concentrations of unlabeled cyclo(His-Pro) as described in Methods.

Fig. 4. Thin-layer chromatography of membrane-bound radioactivity, [^{3}H]cyclo(His-Pro) and authentic cyclo(His-Pro). As described in Methods, membrane-bound cyclo(His-Pro) was collected. This membrane-bound radioactivity, as well as [^{3}H]cyclo(His-Pro) and authentic cyclo(His-Pro), was applied to a silica gel plate and developed in ascending mode. The plates were cut, extracted and counted for radioactivity as described in Methods. Authentic cyclo(His-Pro) was visualized by the Pauly reaction.

competitive results yielded a slope corresponding to a dissociation constant of 59.0 nM and a maximal binding of 4.6 pmol/mg protein as shown in Fig. 3. The specificity of the binding reaction was shown by the observation that 3000 more TRH than cyclo(His-Pro) was required for displacement of bound [^{3}H]cyclo(His-Pro). The specificity of the binding is likely to depend on the histidine than the proline moiety of cyclo(His-Pro) because proline in the dose of 4500 μM did not compete for the binding of [^{3}H]cyclo(His-Pro), while histidine was effective with an IC_{50} of 545 μM.

Characteristic of Bound Cyclo(His-Pro). As shown in Fig. 4, the thinlayer chromatography showed that [^{3}H]cyclo(His-Pro) after exposure to liver membranes was identical to authentic dipeptide, indicating the absence of metabolism of cyclo(His-Pro) during the course of interaction with membranes.

Table 3. Effects of cyclo(His-Pro) and TRH administration to rats on specific binding and affinity constance of [^{3}H]cyclo(His-Pro) binding in liver membranes

Group	N	Specific binding (fmol/mg protein)	Affinity constance (Kd= nM)
Saline	(5)	96.9 ± 10.8	77.3 ± 14.8
Cyclo(His-Pro)	(5)	58.7 ± 8.1*	68.8 ± 9.8
TRH	(5)	72.2 ± 13.1	70.2 ± 12.3

As described in Methods, specific binding of [^{3}H]cyclo(His-Pro) was measured in liver plasma membranes from adult male rats to which either saline, cyclo(His-Pro) or TRH was administered. The data are presented as mean ± S.E.M. The statistical analysis was done by Student's t test (*P<0.05, cyclo(His-Pro) vs saline).

Therefore, the binding of cyclo(His-Pro) apparently indicates a recognition of the structure of the original dipeptide.

Effects of In Vivo Administration of Cyclo(His-Pro) and TRH on Cyclo(His-Pro) Binding in Liver Membranes. Whereas administration of TRH did not cause any changes in cyclo(His-Pro) binding, injection of cyclo(His-Pro) significantly decreased the specific binding numbers, but not the binding affinity, as shown in Table 3.

DISCUSSION

We have identified and characterized binding for cyclo(His-Pro) in crude plasma membranes from the rat liver which interacted with cyclo(His-Pro) in a specific and saturable manner. In the adrenal gland, membrane binding of cyclo(His-Pro) was reported to be relatively low affinity (Kd=900-1800 nM) in nature (10,11). In contrast, the present study demonstrated that plasma membranes derived from the rat liver bound cyclo-(His-Pro) with high affinity (Kd=59 nM). This value of Kd was nearly comparable to that of beta-endorphin binding in the rat liver membranes (Kd=53 nM), where beta-endorphin was observed to stimulate adenylate cyclase activity in a dose-related fashion

(16). Because cyclo(His-Pro) was reported to increase cyclicAMP and cyclicGMP levels in the rat cerebelli (17), cyclo(His-Pro) showing high affinity binding in liver membranes has been expected to possess the pivotal biological significance in the liver. If so, since the liver membranes were reported to display low affinity binding (Kd=5 μM) for TRH, a possible precursor of cyclo(His-Pro) (13), it may be possible that TRH has the physiological implication in the liver after converted to cyclo(His-Pro). Now the studies are in progress to elucidate whether cyclo(His-Pro) may stimulate cyclic nucleotides in the rat liver cells.

The specific binding of cyclo(His-Pro) in liver membranes was significnatly reduced by in vivo injection of cyclo(His-Pro), but not TRH. A number of receptor bindings of bioactive peptides and hormones have been shown to have either downregulation or upregulation by themselves (9,18), the present results showing the apparent downregulation of cyclo(His-Pro) binding sites indicate the certain existence of a receptor-like characteristic of cyclo(His-Pro) binding in the rat liver plasma membranes.

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