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HISTIDYL-PROLINE DIKETOPIPERAZINE, AN ENDOGENOUS BRAIN PEPTIDE THAT INHIBITS ($Na^+ + K^+$)-ATPase

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Summary: Histidyl-proline diketopiperazine, a metabolite of thyrotropin releasing hormone, has been detected in brain and has a variety of biological activities, both in the pituitary and the central nervous system. An investigation of the biochemical basis for the physiological actions of the peptide has shown that it is an inhibitor of dopamine uptake in nerve endings. The inhibition by the peptide of catecholamine transport, which is sodium-dependent, is due to an inhibition of the brain plasma membrane ($Na^+ + K^+$)-ATPase. These findings suggest that histidyl-proline diketopiperazine may be an inhibitor of many membrane transport processes dependent on ion gradients.

Introduction: Histidyl-proline diketopiperazine[1] is a unique cyclic dipeptide which is formed by metabolism of TRH (1-3). Tracer studies have demonstrated that injection of TRH into rat brain leads to the accumulation of radioactive cyclo(His-Pro) (2). It has been established that this cyclic dipeptide is a biologically active agent. In addition to a role in pituitary function (inhibition of prolactin release (4, 5)), it also has activity in the central nervous system; the peptide antagonizes ethanol sedation (2), produces hypothermia (6) and elevates the concentration of cGMP in rat brain (7).

Dopamine has been implicated with a number of the processes mentioned above (8-10 We therefore tested the possibility that the mechanism of action of cyclo(His-Pro) might involve some aspect of catecholamine metabolism. As an approach to this questio we studied the effect of cyclo(His-Pro) on dopamine uptake.

The studies presented here established that cyclo(His-Pro) is an inhibitor of dopamine uptake in rat brain striatal synaptosomes. The mechanism for inhibition of dopamine uptake by the peptide involves a specific inhibition of the ($Na^+ + K^+$)-ATPase

Materials and Methods: Dopamine uptake into rat striatal synaptosomes was carried out by the procedure detailed in the legend to Fig. 1A. ATPase activity in synaptosome membranes was measured as described in Table 1 as release from ATP of Pi or by a spectrophotometric assay coupled to the oxidation of DPNH (see legend to Fig. 2). [^{3}H]-Ouabain and [^{48}V]-vanadate binding to synaptosome membranes were measured as described in the legends to Figs. 3A and 3B, respectively.

1. Abbreviations used: histidyl-proline diketopiperazine, cyclo(His-Pro); thyrotropin releasing hormone, TRH.

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RESULTS

Cyclo(His-Pro) Inhibits Dopamine Uptake: We studied the effect of cyclo(His-Pro) on the uptake of dopamine into synaptosomes prepared from rat striatum. In the synaptosome preparations used, dopamine uptake was proportional to incubation time up to approximately 3 min (data not shown). The data presented here are for incubation times of 1 min and therefore correspond to initial rates of dopamine uptake. In control incubations, the uptake (80 pmoles/mg of protein/min), was similar to that in previous reports (11). As shown in Fig. 1A, cyclo(His-Pro) (2) inhibits dopamine uptake (50% inhibition at approximately 300 μM). A comparative test of the effect of TRH (Calbiochem) on dopamine uptake shows that the releasing hormone, tested at concentrations up to 10^{-3} M, has no effect on catecholamine uptake.

The kinetics of the process was studied (14). In agreement with previous studies (15), a Lineweaver-Burk analysis of the effect of d-amphetamine on dopamine uptake (Fig. 1B, panel A) suggests that this drug probably competes directly (16) with the catecholamine for binding to the transporter.

In contrast, cyclo(His-Pro) is a noncompetitive inhibitor of dopamine uptake (Fig. 1B, panel B), suggesting an indirect action. Ouabain is also a noncompetitive inhibitor of dopamine uptake (11) and is thought to inhibit dopamine uptake indirectly by inactivating the membrane-bound sodium pump (11), since the accumulation of this amine is dependent on sodium and potassium (17). We therefore considered the possibility that cyclo(His-Pro) inhibits the ion pump.

Cyclo(His-Pro) Inhibits $(Na^+ + K^+)$-ATPase: Fragments of striatal synaptosome membranes were prepared by freezing and thawing synaptosomes (11) and used for the measurement of ATPase activity (Table 1). A concentration of cyclo(His-Pro) (500 μM) which produces approximately 70% inhibition of dopamine uptake (Fig. 1A) was observed to inhibit total ATPase (assayed in presence of Na^+, K^+ and Mg^{++}) in striatal synaptosome membranes to the extent of 42%. The ATPase activity measured in the absence of added Na^+ and K^+ was 17% inhibited by cyclo(His-Pro). A calculation of the $(Na^+ + K^+)$-specific ATPase showed that the specific ATPase was 66% inhibited by cyclo(His-Pro). The similarity in the degree of inhibition by cyclo(His-Pro) of $(Na^+ + K^+)$-ATPase (Table 1) and dopamine uptake (Fig. 1A) suggests that inhibition of ATPase by the peptide is responsible for inhibition of dopamine uptake.

The study detailed in Fig. 2 (panel A) documents the effects of added cyclo(His-Pro) or TRH (both tested at a concentration of 500 μM) on the activity of the $(Na^+ + K^+)$-ATPase at a variety of $MgCl_2$ concentrations. The ATPase activity assayed in the absence of added peptide decreases with increasing concentration of $MgCl_2$ (see legend to Fig. 2, panel A). The Mg^{++}-dependent inhibition is reflected mainly in the $(Na^+ + K^+)$-dependent fraction of the ATPase through the range of concentration 3-12 mM. At higher concentrations of $MgCl_2$, both the Mg-dependent and the $(Na^+ + K^+)$-dependent activities are inhibited. There is no significant inhibition of the ATPase by TRH at any of the concen-

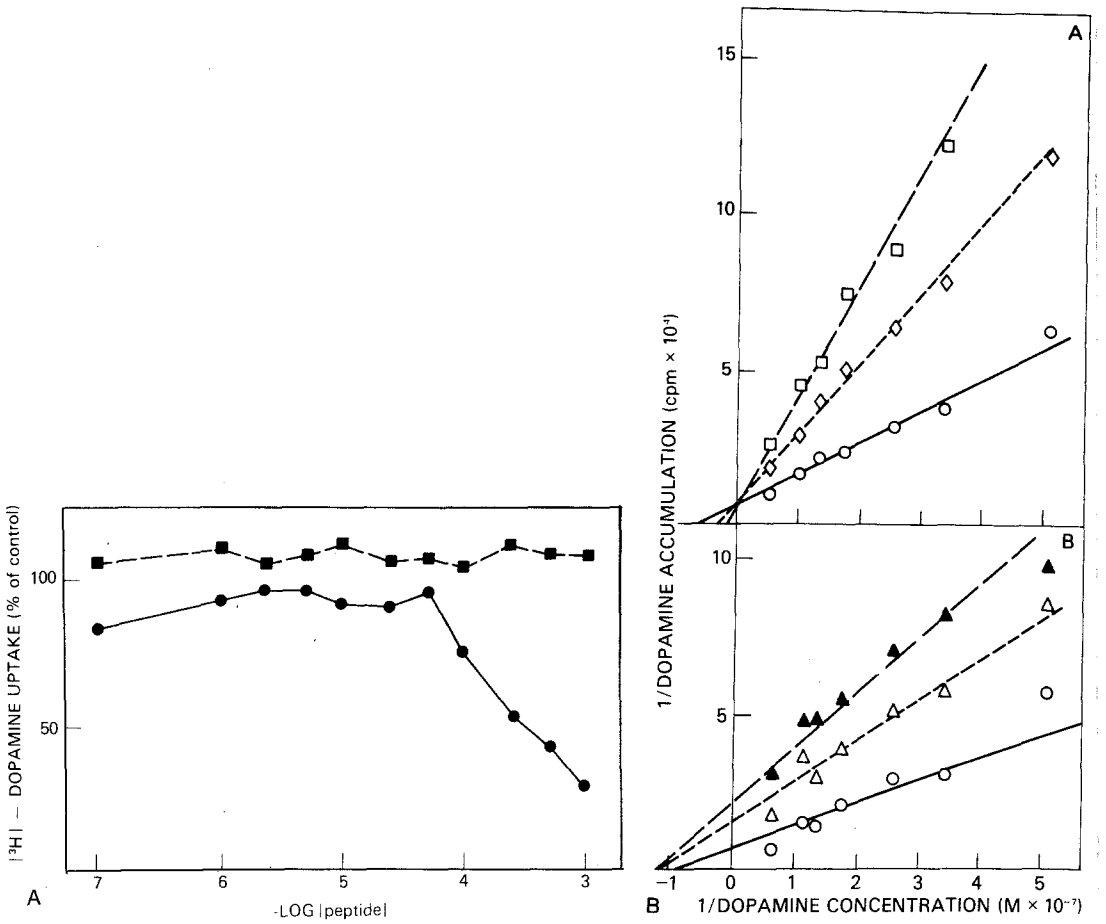

Fig. 1 (A) Effect of concentration of cyclo(His-Pro) (●) and TRH (■) on [^{3}H]-dopamine uptake. Rat striatal synaptosomes (12) were prepared by homogenizing (Teflon-glass homogenizer, 10 strokes by hand) the striatum from one male Sprague-Dawley rat (200 g) in 20 vol of ice-cold 0.32 M sucrose; after sedimentation of the nuclear fraction (1000 x g for 10 min), the supernatant solution was centrifuged at 27,000 x g for 15 min. The pellet (P_2 fraction) was resuspended in 20 vol of ice-cold 0.32 M sucrose (approximately 1.5 mg/ml of protein) (13). For measurements of dopamine uptake, incubation mixtures (500 µl) contained 119 mM NaCl, 3.9 mM KCl, 0.65 mM $MgSO_4$, 0.51 mM $CaCl_2$, 19 mM NaH_2PO_4 (pH 7.4 at 37°C), 0.1 mg per ml ascorbic acid, 10 mM glucose, 0.08 mM pargyline (Sigma) and synaptosomes (30 µg of protein). After preincubation for 5 min at 37°C, [^{3}H]-dopamine (New England Nuclear, specific activity 6.3 Ci/mmol, 0.1 µM) was added and incubation was continued for 1 min. The uptake was terminated by pouring 400 µl of the suspension over 0.45 µ Millipore filters and washing with ice-cold 0.9% NaCl (2 x 2.5 ml). The filters were then dried and counted. Blanks were carried out as above with a 5 min preincubation at 37°C, but with incubation with [^{3}H]-dopamine for 1 min at 0°C. Results are expressed as percentage of the control activity (∿5000 cpm) corrected for blank incorporation (approx. 500 cpm).
(B) Double reciprocal plot of dopamine uptake in absence (○) or presence of A) d-amphetamine 10^{-7} M (◇), 2.5 x 10^{-7} M (□) and B) cyclo(His-Pro) 10^{-4} M (△), 2.5 x 10^{-4} M (▲). Dopamine uptake was measured using various concentrations of [^{3}H]-dopamine (10^{-8}-10^{-7} M) and the indicated concentrations of d-amphetamine (Sigma) or cyclo(His-Pro).

trations of $MgCl_2$ tested. However, the degree of inhibition of the ATPase by cyclo-(His-Pro) is dependent on the $MgCl_2$ concentration; while 40% inhibition of ($Na^+ + K^+$)

Table 1

Inhibition of ATPase by Cyclo(His-Pro)

ATPase activity (μmoles/mg protein/hr)

Additions	Control	Cyclo(His-Pro)	% Inhibition
$Na^+ + K^+ + Mg^{++}$	13.58	7.81	42
Mg^{++}	6.51	5.40	17
$Na^+ + K^+$	7.07	2.41	66

Synaptosomes prepared as described in Fig. 1 were frozen and thawed then diluted with an equal volume of 1 M KCl. After washing twice by centrifugation at 20,000 x g for 20 min with 0.32 M sucrose, the final pellet was resuspended at a total concentration of 400 μg protein/ml and stored in 0.5 ml aliquots at -20°C (11). For assay of ATPase, incubation mixtures contained 3 mM ATP (Tris) (Sigma), 3 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.4), 90 mM NaCl, 10 mM KCl and 10 μg of synaptosome protein per ml. For measurement of Mg^{++} ATPase, the NaCl and KCl were omitted. The reaction was carried out for 15 min at 37°C and stopped by adding trichloroacetic acid to a final concentration of 6%. After centrifugation at 1000 x g for 20 min, Pi was measured in aliquots of the supernatant solution according to Dryer et al (18). $(Na^+ + K^+)$-ATPase activity was calculated as the difference between total $(Na^+ + K^+ + Mg^{++})$ and (Mg^{++})-ATPase.

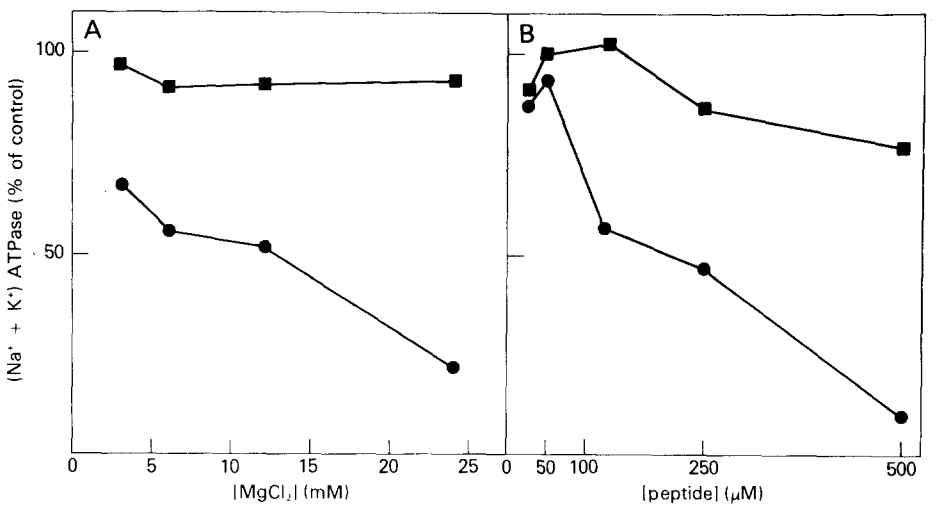

Fig. 2 Effect of concentration of $MgCl_2$, TRH and cyclo(His-Pro) on $(Na^+ + K^+)$-ATPase in synaptosome membranes. ATPase activity was measured spectrophotometrically at 37°C with a coupled assay (hydrolysis of ATP coupled to oxidation of DPNH using pyruvate kinase and lactate dehydrogenase) (19). Incubation mixtures (1 ml) contained: 90 mM NaCl, 30 mM Tris-HCl (pH 7.4), 25 mM KCl, 25 mM $MgCl_2$ except where otherwise indicated, 3 mM ATP (Tris, vanadium-free), 0.4 mM DPNH, 0.5 mM PEP, 0.02% BSA, approximately 3 units of pyruvate kinase, 4 units of lactate dehydrogenase and synaptosomes (10 μg of protein). The oxidation of DPNH was followed in a Gilford automatic recording spectrophotometer at 340 nm. The $(Na^+ + K^+)$-ATPase activity was determined by subtracting the total activity from that obtained by assay in the presence of 10^{-3} M ouabain. Data are expressed as a percentage of the control activities. A: $MgCl_2$ concentration was varied from 3 to 24 mM in incubation mixtures deficient in or supplemented with TRH (500 μM) (■) or cyclo(His-Pro) (500 μ) (●). The total and ouabain inhibited activites, respectively, were (in μmoles DPNH oxidized/mg protein/hr): 3 mM $MgCl_2$, 28.83, 12.46; 6 mM $MgCl_2$, 26.79, 13.95; 12 mM $MgCl_2$, 21.58, 10.79; 24 mM $MgCl_2$, 13.02, 7.44. B: Peptide concentration (either cyclo(His-Pro) or TRH) was varied from 25 to 500 μM. The control activity in the absence of added peptide was 7.08 μmoles DPNH oxidized/mg protein/hr. (■), TRH; (●), cyclo(His-Pro).

ATPase is observed at 3 mM $MgCl_2$, 80% inhibition is obtained at 24 mM $MgCl_2$. A study of the effects of dose of TRH or cyclo(His-Pro) on the activity of $(Na^+ + K^+)$-ATPase (Fig. 2, panel B) showed that 50% inhibition of the activity was obtained at 250 µM diketopiperazine, while concentrations of TRH as high as 500 µM had little, if any, effect on the $(Na^+ + K^+)$-ATPase. Therefore, the cyclic dipeptide-dependent inhibition of $(Na^+ + K^+)$-ATPase is specific for the diketopiperazine structure and depends on the concentration of Mg^{++}.

Mechanism of Cyclo(His-Pro)-dependent Inhibition of $(Na^+ + K^+)$-ATPase: Since ouabain and the cyclic dipeptide show noncompetitive inhibition of dopamine uptake (Fig. 1B, panel B) and inhibit the $(Na^+ + K^+)$-specific fraction of the synaptosome ATPase (Table 1 and Fig. 2), we considered the possibility that cyclo(His-Pro) binds specifically to the unique ouabain binding site (20) of the ATPase. Under the conditions described in Fig. 3A, synaptosome membrane fragments bound 0.17 pmoles of [^{3}H]-ouabain to 10 µg of membrane protein; binding of [^{3}H]-ouabain was effectively displaced by unlabeled ouabain (50% displacement at approximately 8 x 10^{-8} M). However, cyclo(His-Pro) tested at concentrations between 10^{-9}-10^{-3} M, did not compete for the binding of [^{3}H]-

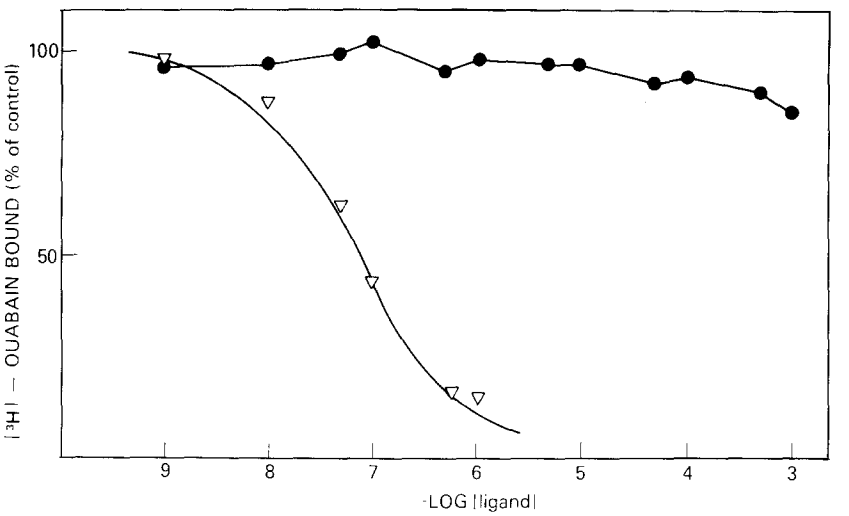

Fig. 3 (A) Effect of cyclo(His-Pro) on [^{3}H]-ouabain binding to synaptosome membranes. Synaptosome membranes were prepared as described in Table 1. For measurement of [^{3}H]-ouabain binding (21), incubation mixtures (total volume 95 µl) contained: 52 mM Tris-HCl (pH 7.4), 4.7 mM ATP (Tris, vanadium-free), 4.7 mM $MgCl_2$, 4.7 mM EGTA, 95 mM NaCl and [^{3}H]-ouabain (New England Nuclear) 1.7 x 10^{-8} M (0.04 µCi); the indicated concentrations or either ouabain (▽) or cyclo(His-Pro) (●) were included in the incubation mixtures. The binding was started by adding synaptosomes (10 µg of protein). After incubation for 30 min at 37°C, the reaction was stopped by filtering 70 µl through a Millipore filter (0.45 µ), following the same procedure as indicated for [^{3}H]-dopamine uptake (see Fig. 1). Specific binding was determined by subtracting the non-specific binding (in presence of 10^{-3} M unlabeled ouabain). A control incubation containing no added unlabeled ouabain or cyclo(His-Pro) bound 3917 cpm while the non-specific binding was 183 cpm. The data are expressed as a percentage of the corrected activity of the control incubation mixture.

ıabain to the membrane fragments. The conditions for ouabain binding used here involve ıe absence of K^+, since K^+ inhibits the binding of ouabain (23). In data not shown, : found that whether ouabain binding was performed in the presence or absence of added (10 mM), the diketopiperazine was ineffective in displacing bound ouabain. We conclude ıat the inhibition of ATPase by cyclo(His-Pro) does not involve the ouabain binding site.

We tested the idea that cyclo(His-Pro) inhibits ATPase by a mechanism similar to ıat of vanadate. [^{48}V]-Vanadate binding (22) was measured in synaptosome membrane :agments. Under the conditions described in Fig. 3B, the membranes bound 0.06 pmoles : [^{48}V]-vanadate to 10 µg of membrane protein; the binding of [^{48}V]-vanadate was :fectively displaced by unlabeled vanadate (50% displacement at 4×10^{-8} M). In ıpport of the concept that vanadate binds to a low affinity ATP site, we observed ıat 50% displacement of [^{48}V]-vanadate required in excess of 10^{-3} M ATP. Most ignificantly, cyclo(His-Pro) diminished vanadate binding more effectively than did ATP 50% displacement at approximately 10^{-4} M). The competition for vanadate binding ppeared to be quite specific for cyclo(His-Pro); TRH, cyclo(His-Gly), cyclo(Gly-Pro), -histidine and L-proline tested at 10^{-3} M produced no displacement of [^{48}V]-vanadate.

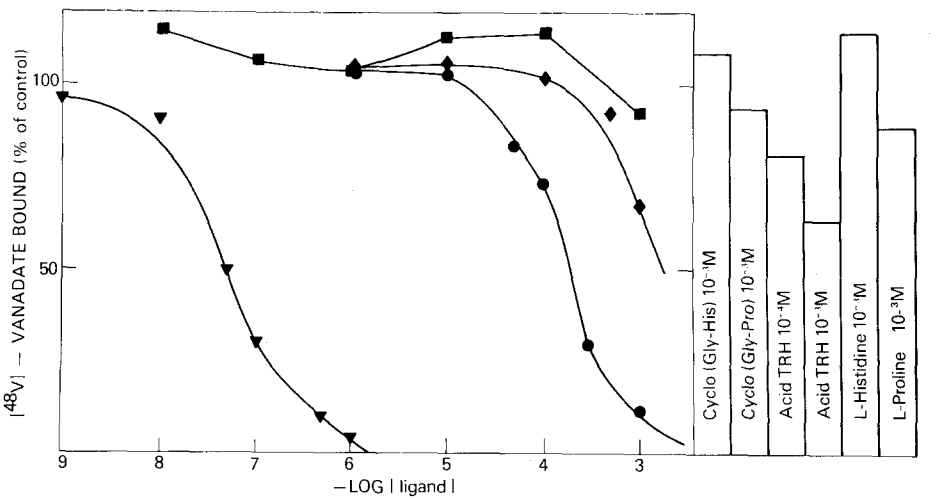

Fig. 3 (B) Effect of cyclo(His-Pro) and related compounds on [^{48}V]-vanadate binding. Synaptosome membranes were prepared as described in Table 1. [^{48}V]-Vanadate binding (22) was assayed in a total volume of 100 µl containing 25 mM $MgCl_2$, 25 mM KCl, 20 mM Tris-HCl (pH 7.4), [^{48}V]-vanadate, 10 nM (0.04 µCi) and the indicated concentration of various compounds: vanadate (Fisher) (▼), cyclo(His-Pro) (●), ATP (◆), TRH (■) and other related compounds, shown in the bars. The incubation was initiated by adding synaptosomes (10 µg of protein). After incubation for 60 min at 37°C, 80 µl aliquots were filtered and washed as described in Fig. 1.[^{48}V]-vanadyl chloride was from Amersham. After dilution with H_2O to the desired concentration and neutralization with 1 N NaOH, it was incubated at 25°C for 3 hours before using. Specific binding was determined by subtracting the non-specific binding obtained in the presence of added 10^{-4} M unlabeled vanadate. A control incubation containing no added unlabeled ligand bound 5300 cpm, while the non-specific binding was 210 cpm. The data are expressed as a percentage of the counts in the control corrected for non-specific binding. Cyclo(Gly-His), cyclo-(Gly-Pro) and acid TRH were from Chemical Dynamics, South Plainfield, New Jersey.

Acid TRH (pGlu-His-Pro) produced some displacement of [^{48}V]-vanadate but was at least 10 times less potent than cyclo(His-Pro) (Fig. 3B). These studies suggest that cyclo-(His-Pro) inhibits synaptosomal ($Na^+ + K^+$)-ATPase with a site of action at the vanadat binding region.

DISCUSSION

($Na^+ + K^+$)-stimulated ATPase is present in high concentration in brain and is distributed in various cell types (24). The enzyme, which is concentrated in nerve terminals (25), plays an important role in neurons of maintaining ion gradients necessary for nerve impulses (20). In glia, the ATPase functions in regulating the extracellular K^+ concentration. In both neurons and glia, a variety of transmitter transport systems depend indirectly on the enzyme for a source of extracellular Na^+ which is co-transported with the substrate (26). The finding that cyclo(His-Pro) inhibits dopamine uptake and ($Na^+ + K^+$)-ATPase in crude synaptosome fragments opens the possibility that the peptide may have a regulatory function in any number of these cell-types or processes.

Endogenous factors from skin and brain that inhibit ouabain binding to ATPase have recently been described (27-29). The studies presented here show that cyclo-(His-Pro) inhibition of ($Na^+ + K^+$)-ATPase does not involve the ouabain binding site. Therefore, cyclo(His-Pro) functions by a different mechanism than these other factors.

Vanadium (in the 5^+ oxidation state) has received a great deal of attention recently as a factor that regulates the activity of ($Na^+ + K^+$)-ATPase (22, 30). Vanadium is found in muscle and brain in a concentration range of 10^{-6}-10^{-7} M (31) and is an important dietary factor for rodents (32). Vanadate inhibits ($Na^+ + K^+$)-ATPase from kidney (33), red cells (34), and brain (35) (sodium dodecyl sulfate extrac of microsomes) in the nanomolar range, by binding to a low affinity ATP binding site (22). We have found that vanadate also inhibits the enzyme from synaptosome fragments (50% inhibition of the total activity at 0.5 μM; data not shown), and binds to the preparation with high affinity (Fig. 3B). Inhibition of dog kidney ($Na^+ + K^+$)-ATPase by vanadate is dependent on Mg^{++} (35). The observation that the inhibition of ($Na^+ + K^+$)-ATPase by cyclo(His-Pro) was increased with increasing concentrations of Mg^{++} is consistent with the interpretation that binding of cyclo(His-Pro) to the vanad site is the locus for peptide-dependent inhibition of ($Na^+ + K^+$)-ATPase. The vanadate site (a low affinity binding site for ATP) has been proposed to be on the inner surfac of the plasma membrane (34), which suggests that the site of action of cyclo(His-Pro) also on the cytoplasmic side of the cell. In contrast, ouabain inhibits the ATPase by interaction with a site on the extracellular side of the enzyme.

In summary, the studies presented here have demonstrated that cyclo(His-Pro) is a endogenous peptide which acts as a highly specific inhibitor of brain ($Na^+ + K^+$)-ATPas

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