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The Effects of the TRH Metabolite Cyclo(His-Pro) and Its Analogs on Feeding

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KOW, L -M AND D W PFAFF. *The effects of the TRH metabolite cyclo(His-Pro) and its analogs on feeding* PHARMACOL BIOCHEM BEHAV **38**(2) 359–364, 1991 —Cyclo(His-Pro), or cHP, is a putative metabolite of thyrotropin-releasing hormone (TRH), and, like TRH, can inhibit food intake but requires higher doses In attempts to improve the anorectic effects of cHP through modification of its structure, a number of its analogs were synthesized These analogs or cHP itself were administered to rats either by intracerebroventricular (ICV) infusion or systemic injection, and their effects on food intake were measured None of the synthetic analogs was more potent than cHP, although several analogs demonstrated comparable potencies to the parent compound Interestingly, one cHP analog reversed the suppressive effect and stimulated feeding This reversal, as well as the preservation of the anorectic effect by some but not all the analogs, suggests that the cHP effect on feeding does require specific structural features

Cyclo(His-Pro) Thyrotropin-releasing hormone Food intake

CYCLO(HIS-PRO), or histidyl-proline diketopiperazine, is a metabolite of thyrotropin-releasing hormone (TRH). Both cyclo(His-Pro), or cHP, and TRH are widely distributed in the brain in high concentrations (7,8) and share several biological actions [cf (15)] including the regulation of feeding. Earlier reports have shown that cHP, like TRH (10, 17–19), inhibits food intake when administered by intracerebroventricular (ICV) infusion to rats (10, 11, 20). Furthermore, the level of hypothalamic cHP appeared to be associated with feeding states· elevated during fasting and returning to normal upon feeding (9). These findings suggest endogenous cHP could be involved in the regulation of food intake. However, the anorectic effect of cHP has been questioned by two recent studies (1,6) These two studies, conducted in the same laboratory, showed that cHP at doses ranging from 14 to 1000 nmol/rat, ICV, had no effect on spontaneous or 24-hour starving-induced feeding. In contrast, earlier reports showed that cHP at 10 to 1000 nmol/rat, ICV, inhibited spontaneous, tail pinch-induced, 24-hour starving-induced, or norepinephrine-induced feeding (10, 11, 20). Although there are some differences in methods, such as types of testing pellets, testing cages, testing schedules, etc., between the earlier and the recent studies, it is not known whether such methodological differences can account for the discrepancy in results In pilot experiments (Kow et al., unpublished data), we found that ICV infusion of cHP into rats consistently inhibited food intake, but only at the relatively high doses of 1 and 2 μmoles/rat. Although the reasons for requiring such high doses are not understood, cHP may be acting by modulating other peptides or neurotransmitters as suggested by an electrophysiological study (4). Alternatively, the structure of cHP molecule may not be optimal and can be modified to improve potency. To investigate this latter possibility, a number of synthetic analogs were prepared and tested via ICV administration in various feeding paradigms.

Although cHP shares some biological and behavioral actions with TRH, it does not bind to TRH receptors (2, 3, 16). In fact, no specific binding for cHP could be found in the brain (3). Therefore, in the modification of cHP, strategies for developing TRH analogs were not adopted. Instead, in some cases, the parent cHP molecule was modified to increase the lipophilicity. Such a modification would increase anorectic potency by improving passage into the brain and/or cell membrane, if cHP acts by simply dissolving in the neuronal membrane to alter membrane characteristics, such as fluidity. In other cases, the size and shape of the rings or side chains of the parent molecule were altered in attempts to improve potency. Such alterations also enable us to see whether there are structural constraints upon cHP action If the action is mediated through a receptor, some structural requirement should exist.

EXPERIMENT 1. INTRACEREBROVENTRICULAR (ICV) INFUSION

To compare the potencies of cHP and its analogs, the compounds were initially administered at suprathreshold doses to establish anorectic activity. Those compounds possessing activity were then tested at lower doses to establish a minimum effective dose to achieve a significant anorectic response. All test agents were administered through ICV infusion.

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METHOD

Subjects

Adult, male Sprague-Dawley rats were used and housed individually in an air-conditioned room with lights on from 8:00 a.m. through 8.00 p.m. The animals were given free access to rat chow and water (unless otherwise indicated) and allowed to adapt to the experimental environment for at least one week before testing. The rats were tested in squads of 16 to 24.

Cannula Implantation

The rats were anesthetized with Chloropent (0.25 ml/100 g body weight), and implanted in the skull with a guide cannula whose tip was aimed at the right lateral ventricle. The cannula was made from a 22-gauge hypodermic needle, which was cut to a length such that, when implanted, the tip of the cannula would be 3.7 mm below the dorsal surface of the skull. With the skull leveled between the bregma and the designated lambda (13), the cannula was implanted at 0.5 mm posterior to the bregma and 1.5 mm lateral to the midline. The plastic hood of the hypodermic needle was trimmed and filled with a medical silicone glue (Dow Corning). The glue seals the cannula but allows the insertion of the inner cannula for infusion and prevents leakage of test solution upon the withdrawal of the inner cannula. After the surgery, the rats were undisturbed for one week.

Experimental Procedure

After recovery from surgery, the rats were trained daily to a mild food deprivation, a handling/infusion, and a feeding test. The food deprivation lasted from 9:00 a.m. to the time of feeding test at 2:00 p.m. Twenty minutes before the feeding test each rat received handling and a sham ICV infusion. The rats were presented with a milk diet (condensed sweetened milk by Borden, and water, 1:1) for 30 min. Pilot experiments demonstrated that rats did not drink water during the test period. At the end of the 30-min test period, the milk was removed for measurement and ad lib supply of water and rat chow was reinstated. This training protocol continued for 7–10 days until food intake and other behavioral paradigms (such as latency to feed, pattern of feeding, etc.) became stabilized. On testing day, cHP or an analog was infused ICV (see below). Subsequently, rats were tested daily without further experimental treatment to monitor their return to pretreatment level and then (3 or more days) tested with a different agent.

To perform an ICV infusion, the rat was gently held by an experimenter, and an inner cannula (gauge 28) connected to tubing and a microsyringe was inserted through the guide cannula to infuse 10 μl of a test solution. The infusion was performed over 60 s with the inner cannula remaining in place for an additional 30 seconds to allow the infusate to diffuse.

To verify the validity of cannula location, each rat was given an ICV infusion of angiotensin II (50 ng/5 μl saline/over 30 s plus a 30-s wait) following the end of the feeding test. Pilot experiments with a combination of the angiotensin infusion followed by postmortem examination of an ICV-infused dye demonstrated that the rats with correct cannula placement were those that drank water within two minutes after the angiotensin infusion. Therefore, rats that did not do so upon angiotensin administration were considered to have invalid cannula placement, and their results were excluded from data analysis.

Test Agents

cHP (Sigma) and thirteen cHP analogs synthesized by Abbott Laboratories were tested. The analogs were synthesized with either the proline or the imidazole ring modified. The structure and designated A-number for all the analogs synthesized and tested are presented in Table 1. The test agent was dissolved in a vehicle to a concentration of 2 μmoles/10 μl for screening tests, or lower, for dose-response tests. The water-soluble analogs were dissolved in a combination of saline and distilled water to maintain an isotonic solution. The water-insoluble analogs were dissolved in propylene glycol (PG), or, in one case, in 25% dimethyl sulfoxide (DMSO) in saline. The vehicle used for each analog is also listed in Table 1.

Measurements and Observations

The absolute milk consumption by each rat was determined by measuring the difference between pre- and posttest milk volumes to the nearest 0.5 ml. Since large individual differences in milk consumption were observed, the measurements were normalized by expressing milk consumption on treatment day as a percentage of the baseline consumption level obtained from a given rat in the last 3 days of training. These normalized percentage figures were used to calculate group means. To assess the effect of a particular agent, the mean of the group of rats treated with the agent was compared to that of the appropriate control (vehicle-infused) group with *t*-test.

After each infusion of a test agent, the rat was observed for any abnormal or unusual behavior and for the stereotyped behavioral routine of satiation (grooming, retreating to the back of the cage, and resting/sleeping).

RESULTS

Screening Tests

Effects of cHP. The effects of ICV infusion of vehicle solvents and various agents are summarized in Table 1. Infusion of saline, propylene glycol (PG), or 25% DMSO had no effect on milk consumption. The infusion of cHP, dissolved in saline or PG, reduced milk intake to 48% or 59% of the pretreatment baseline level, respectively. These reductions of milk intake, or anorectic effects, are statistically significant as compared to the results of respective vehicle infusions (Table 1).

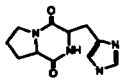
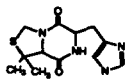
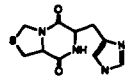
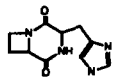
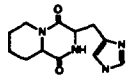
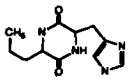
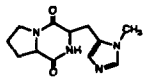
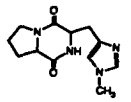
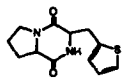
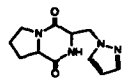
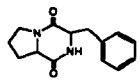
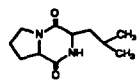
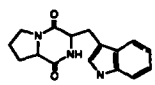
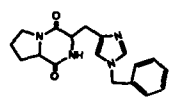
Effects of proline modification. Five analogs were tested that contained a modification in the proline moiety. Introduction of a sulfur in the pyrrolidine ring, with or without additional dimethyl substituents at the 4-position, produced analogs (A-65286, A-65206) that were equipotent to cHP. Contraction of the pyrrolidine to the 4-membered ayzetidine ring also yielded an active analog (A-65171). In contrast, expanding the pyrrolidine ring to the six-membered piperidine homolog (A-65218) abolished the anorectic effects. Likewise, opening the pyrrolidine ring by incorporating norvaline in place of proline also resulted in an inactive analog (A-65177) (Table 1).

Effects of histidine modifications. Modifications at the histidine moiety focused on either substituting or altering the imidazole ring in the side chain. Those analogs that maintained anorectic potencies comparable to cHP include N-methylation of the imidazole at either the 1- or 3-positions (A-67244, A-64705) or replacement of the imidazole with a thiophene (A-65916) or pyrazole (A-65914). In contrast, substituting the imidazole with a phenyl (A-64863), an aliphatic group (A-65190), or an indole (A-64706) produced analogs devoid of activity (Table 1).

Interestingly, attaching a bulky benzyl group to imidazole 3-nitrogen produced an analog (A-65913) that reversed the anorectic effect, causing the rats to consume more milk than their

TABLE 1

TEST AGENTS THEIR CHEMICAL STRUCTURES AND EFFECTS ON MILK CONSUMPTION

Agents	Chemical Structure	Solvents	No of Rats	Milk Consumption* (% of Control)	p†
		Saline	29	103 4 ± 3 2	
		25% DMSO	3	96 1 ± 19 0	
		PG‡	15	102 6 ± 5 3	
cHP		Saline	9	48 0 ± 7 1	<0 001
cHP		PG	5	59 3 ± 7 9	<0 001
Proline Modifications					
A-65286		PG	9	44 2 ± 4 8	<0 001
A-65206		Saline	6	55 9 ± 8 5	<0 001
A-65171		Saline	6	57 5 ± 5 1	<0 001
A-65218		Saline	7	93 9 ± 6 4	NS
A-65177		Saline	6	104 5 ± 5 4	NS
Histidine Modifications					
A-67244		Saline	8	51 2 ± 7 8	<0 001
A-64705		Saline	13	58.5 ± 5 0	<0 001
A-65916		PG	6	59 9 ± 2 7	<0 001
A-65914		PG	5	63 4 ± 9 5	<0 05
A-64863		25% DMSO	8	99.6 ± 7 9	NS
A-65190		PG	5	100 0 ± 14 0	NS
A-64706		PG	5	106 1 ± 12 0	NS
A-65913		PG	8	>142 2 ± 12 1	<0 01

*Milk consumption (M.C) = (Postinfusion M C /Control M C) × 100%

†p Values are two-tailed for t-test against corresponding solvent

‡PG = propylene glycol

TABLE 2

RESULTS FROM FEEDING-INDUCING TESTS WITH A-65913

	Experimental	Control	p (2-tailed)
Preinfusion			
% of rats consuming milk	100	100	NS*
ml of milk consumed, mean ± SEM (n)	8 1 ± 1 1 (7)	9 0 ± 0 7 (5)	NS†
Postinfusion			
% of rats consuming milk	100	60	NS*
ml of milk consumed, mean ± SEM (n)	7 6 ± 1 1 (7)	0 9 ± 0 4 (5)	<0 001†

*Fisher exact probability tests, Experimental vs Control
†t-Tests, Experimental vs Control

baseline levels (Table 1). To ensure that this surprising result was replicable, a modified feeding study was conducted on a separate group of rats. The procedure was similar to the previous tests except the rats were not food-deprived and the milk was presented 30 min before through 50 min after ICV infusion Milk consumption was measured twice, immediately before and 50 min after the ICV infusion The results from this test demonstrated that even after the rats were satiated with their regular diet plus milk, ICV infusion of A-65913, but not the vehicle, induced further feeding (Table 2).

Dose-Response Tests

cHP and 6 anorectic analogs were tested at decreasing doses of 1.0, 0.5, 0.25, and 0.1 μmole/rat. The results summarized in Table 3 show that food intake reduction was replicated in a robust fashion. None of the agents tested was anorectic at doses below 0.5 μmole/rat. Also, none of the analogs tested was more effective than cHP itself as an anorectic agent both in inhibiting food intake and in the minimum dose required (Tables 1 and 3).

General Observations

Following the administration of cHP or its analogs, no unusual behaviors or neurological signs were observed in the test rats. Similar observations have been reported with ICV infusion of higher doses (up to 5 mg/kg) of cHP than those used here (14). After several days of training, the rats would anticipate the presentation of milk at the testing time and would immediately start to drink as soon as it became available. They would then drink for approximately 8 to 15 minutes. The drinking was followed by a satiation routine that consisted of grooming, retreating to the back of the cage, and resting/sleeping. Once resting, rarely did a rat return for more milk within the following 2 h. These behavioral parameters were not affected by the infusion of saline, propylene glycol, or a nonanorectic analog. The infusion of an anorectic agent would shorten the meal duration, but did not affect the latency to drink, the pattern of continuous meal-taking, or the satiation routine.

DISCUSSION

In this experiment we have confirmed previous reports (10, 11, 20) that ICV infusion of cHP does inhibit food intake. However, the dose required to do so is high (0.5 μmole/rat or higher), and the potency of the anorectic effect could not be enhanced by structural modifications of the parent compound.

Modifications that improved the lipophilicity by introducing a hetero-atom (A-65206), adding methyl groups (A-67244 and A-64705), or both (A-65286) did not increase the potency of the anorectic effect. Therefore, the lipophilicity, by itself, does not seem to be the single important feature of cHP's structure.

In TRH, methylation of the imidazole nitrogen at position 3 greatly improved its binding to TRH receptors [see (3)] and its transmitter-like stimulatory action (12). Similar modifications of cHP retained but did not potentiate the anorectic action, as shown by results from A-64705 and A-67244. This is to be expected, because cHP does not bind to TRH receptors (2, 3, 16) or show direct electrophysiological stimulatory action (4). By the same token, these results also suggest that the anorectic effects of cHP and TRH are not mediated through classical TRH receptors.

The present study showed that there are structural constraints for the anorectic effect. Methylation (A-65286, A-67244, and A-64705), introduction of the hetero-atom, S (A-65286 and A-65916), and substitution of the proline or imidazole ring with ones having smaller (A-65171) or equivalent size (A-65916) were tolerated; while substitution with larger (A-65218 and A-64863) or broken rings (A-65177 and A-65190) destroyed the anorectic effect Requirements for structure are further indicated by the conversion of the anorectic A-64705 into the feeding-inducing A-65913, by substituting the methyl group of A-64705 with a bulky benzyl group. These structural requirements suggest that cHP is acting through specific receptors to reduce food intake.

TABLE 3

MILK INTAKE, AS MEAN ± SEM % (N) OF PREINFUSION CONTROL, FOLLOWING INFUSIONS OF cHP AND ITS ANALOGS AT VARIOUS DOSES

	Dose (μmole/rat, ICV)				
Analogs	2 0	1 0	0 5	0 25	0 1
cHP	48 0 ± 7 1(9)§	74 3 ± 8 0(5)‡	87 9 ± 6 0(6)*	105 9 ± 6 6(4)	
A-65171	57 5 ± 5 1(6)†	63 7 ± 9 3(6)§	77 2 ± 3 7(6)‡	94 7 ± 6 8(8)	104 5 ± 3 2(5)
A-64705	58 5 ± 5 0(13)§	83 4 ± 4 7(8)‡	82 1 ± 5 5(5)†	89 6 ± 7 4(6)	
A-65206	55 9 ± 8 5(6)§	81 3 ± 12 3(6)†	102 3 ± 8 8(5)		
A-65916	59 9 ± 2 7(6)§	93 4 ± 6 0(11)	100.1 ± 6 2(5)		
A-67244	51 2 ± 7 8(8)§	113 4 ± 8 8(5)			
A-65914	63 4 ± 9 5(5)*	103 6 ± 7 6(11)			

*p<0 05, †p<0 02, ‡p<0 01, §p<0 001, t-tests against corresponding vehicle controls All p's are 2-tailed

TABLE 4

EFFECTS OF SYSTEMIC, SUBCUTANEOUS INJECTIONS OF TRH, cHP AND cHP ANALOGS ON THE 30-MIN CONSUMPTION OF MILK AND MINI-PELLETS

Agents	Dose (mg/kg b wt)	Food Intake[a] in Mean ± SEM (n) %, Measured With Milk	Mini-Pellets
		Agents Dissolved in Saline	
Saline	—	109 8 ± 5 8(11)	
TRH	10	84 4 ± 11 2(5)*	
cHP	10	83 4 ± 7 0(6)†	
Saline	—		87 7 ± 10 2(6)
TRH	20		61 6 ± 8 4(6)*
		Agents Dissolved in Propylene Glycol (PG)	
PG	—	117 6 ± 5 3(18)	
TRH	10	97 3 ± 4 8(6)*	
cHP	10	105.9 ± 4 8(12)	
A-65914	10	107 2 ± 3 7(6)	
PG	—		103 6 ± 12 3(6)
TRH	15		64 3 ± 5 8(5)*
cHP	15		82.0 ± 8 6(6)
A-65914	18		88 7 ± 7.0(6)

[a]Food Intake (FI) = (Postinjection FI/Preinjection control FI) × 100% (see the Method section for details)

*$p<0$ 05, †$p<0$ 02, two-tailed with t-test against the corresponding vehicle controls.

EXPERIMENT 2 SYSTEMIC ADMINISTRATION

Since application of TRH systemically has been reported as effective in inhibiting food intake (19), we investigated whether systemic application of cHP or an analog would also be effective.

METHOD

Adult, male rats were maintained as described above except no surgery was performed. Agents tested included TRH, cHP, and a cHP analog, A-65914. This particular analog was chosen to assess the importance of lipophilicity in peripheral administration, because it was equipotent to cHP in ICV application, but is more lipophilic than cHP, and hence may pass through the blood-brain barrier more readily. The doses used ranged from 10 to 25 mg/kg body weight (see Tables 4 and 5). The test agents were dissolved in saline or PG, and the solutions or vehicle controls were injected subcutaneously 20 min before presentation of the test diet. Concentrations were adjusted to allow injection volumes of 0 1 ml.

Three variations of the feeding test, using milk, mini-pellets (Bio-Serv), or powdered rat chow (Purina Rodent Lab Chow, No. 5001), were utilized. The feeding test employing milk as the diet is the same as that in Experiment 1 The use of mini-pellets followed a similar protocol to the above study. In tests using powdered chow, rats were allowed access to the food for 7 h daily, from 10·00 a.m. through 5·00 p.m., and the cumulative food consumption was measured at 1, 2, and 7 h after food presentation During the rest of the day, the rats were deprived of food, but not water

RESULTS AND DISCUSSION

As shown in Table 4, both TRH and cHP dissolved in saline and administered at a dose of 10 mg/kg were effective and equipotent in inhibiting milk intake. However, when dissolved in PG at the same doses, TRH became less effective while cHP became inactive (Table 4). Such decreases in potency were also seen in central applications (Table 1), possibly because PG retarded the absorption of the test agents. A-65914 dissolved in PG was not effective upon systemic administration. The experiment was repeated replacing the milk with a less palatable mini-chow which might offer greater sensitivity in detecting anorectic agents. The results remained largely the same; TRH but neither cHP nor A-65914 significantly inhibited the consumption of mini-pellets (Table 4) Another possibility for the lack of effect of cHP and A-65914 may be that they were absorbed too slowly to achieve a sufficient circulating level at the time of behavioral test. To test this possibility, the rats were presented with powdered chow, which requires longer time to consume, and the period of food presentation was extended (see the Method section). Again, only TRH showed marginal suppression of feeding and neither cHP nor A-65914 was effective (Table 5).

TABLE 5

EFFECTS OF SYSTEMIC, SUBCUTANEOUS INJECTIONS OF TRH, cHP, AND cHP ANALOGS ON FOOD (POWDERED RODENT CHOW) INTAKE

Agents Dissolved in PG	Dose (mg/kg b.wt.)	Food Intake[a] in Mean ± SEM %, Measured at 1 h	2 h	7 h
PG	—	93 3 ± 13 1[b]	103 4 ± 16.3	102 5 ± 19.1
TRH	25	48 5 ± 18.0*	63 9 ± 12 5	93 6 ± 6 4
cHP	25	94.3 ± 9 4	93 1 ± 6 1	97 0 ± 6 1
A-65916	25	85.0 ± 11 8	88 9 ± 11 2	90 7 ± 10 8

[a]Food Intake (FI) = (Postinjection FI/Preinjection control FI) × 100% (see the Method section for details)

[b]All groups, n = 4.

*$p<0$ 05, one-tailed, with t-test against PG

These results demonstrated that peripheral administration was not an effective way for cHP to inhibit food intake and that modification of cHP molecule intended to increase lipophilicity did not improve its effect.

GENERAL DISCUSSION

Our results show that central, as compared to systemic, application was much more effective for cHP and its analogs to inhibit food intake. This contrast in effectiveness indicates that the site of the anorectic action by cHP and its anorectic analogs is the brain. The anorectic effect of cHP could be preserved, abolished, or even reversed by various structural modifications on the parent compound. The preservation of the effect by some but not other modifications indicates requirements for particular physical interactions between the peptides and brain cells. Nevertheless, the failure to enhance anorectic potency by structural modifications led us to consider other reasons for the requirement for a high dose. One such reason, that cHP acts on food intake not as a direct transmitter but instead as a neuromodulator, was investigated in a subsequent study (5)

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