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ANTAGONISM OF ETHANOL-INDUCED DECREASE IN RAT BRAIN cGMP CONCENTRATION BY HISTIDYL-PROLINE DIKETOPIPERAZINE, A THYROTROPIN RELEASING HORMONE METABOLITE

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Summary: Intraperitoneal administration of thyrotropin releasing hormone (50 μmol/kg) produced an approximately 2-fold increase in rat brain cGMP concentration within 15 min. Histidyl-proline diketopiperazine, a metabolite of thyrotropin releasing hormone, produced a similar effect, but the response was faster and shorter-lasting. Intraperitoneal administration of ethanol (1.5 g/kg) decreased brain cGMP concentration approximately 50% within 10-15 min; thyrotropin releasing hormone or histidyl-proline diketopiperazine, injected 5 min after ethanol, antagonized the ethanol-induced decrease in cGMP. Antagonism of the ethanol-induced decrease in the cGMP level required 10 μmol/kg of thyrotropin releasing hormone but was observed with 5 μmol/kg of histidyl-proline diketopiperazine. These data suggest that the metabolic conversion of thyrotropin releasing hormone to histidyl-proline diketopiperazine might explain the previous observation that thyrotropin releasing hormone elevated the level of brain cGMP and antagonized the ethanol-induced decrease in brain cGMP concentration.

Introduction: Other workers have reported that intraperitoneal[1] administration of EtOH to rats decreased the level of cGMP in cerebellum and other brain areas (1-3). Administration of TRH (either IV or IP) elevated cerebellar cGMP and antagonized the EtOH-induced decrease in cGMP concentration (4, 5). We have shown that cyclo(His-Pro) is a metabolite of TRH in rodent brain (6). In the present study, we tested the hypothesis that cyclo(His-Pro) can replace TRH in regulating the concentration of cGMP in brain. We demonstrate that cyclo(His-Pro) has an effect on brain cGMP levels similar to that of TRH. Not only does the diketopiperazine elevate basal levels of the cyclic nucleotide, it also antagonizes the EtOH-dependent lowering of brain cGMP concentrations.

Materials and Methods: Male Sprague-Dawley rats (approximately 150-250g) were from Taconic Farms N.Y. TRH was a Calbiochem product. Cyclo(His-Pro) was prepared as described previously (7, 8). [^{3}H]-cGMP was from New England Nuclear. In order to reproducibly determine the level of brain cGMP in large numbers of animals, a procedure was developed whereby the level of cGMP was stabilized so that there was no significant destruction of cGMP during the several hours required to decapitate animals, dissect out the brains and prepare homogenates. Focused microwave irradiation (1.2 kw) in a Litton Menumaster 70/50 which was modified by Medical Engineering Consultants, Lexington Mass. was used to stabilize the level of cGMP

1. Abbreviations used: ethanol, EtOH; histidyl-proline diketopiperazine, cyclo(His-Pro); thyrotropin releasing hormone, TRH; intraperitoneal, IP; intravenous, IV.

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Table 1. Prevention by Microwave Irradiation of Post-Mortem Decomposition of Brain cGMP

Microwave time (sec)	% of cGMP recovered after 7 sec wave treatment
0*	<1
1	<1
2	1
3	4
4	160
5	262
6	109
7	100

Groups of 3 rats (weight 150-250 g) were sacrificed by microwave irradiation for the indicated time (see Methods). The animals were then decapitated and the brains were homogenized and extracted as described in Methods. cGMP levels were determined as described in Methods. The concentration of cGMP recovered after 7 sec microwave treatment was 2.06 pmol/mg protein (see Fig. 1).

*, decapitation with no microwave treatment.

in brain. Table 1 shows the results of a study of the effect of microwave irradiation time on the level of recovered cGMP. In the absence of irradiation or with irradiation times of 3 sec or less, no cGMP was detected. This is probably due to destruction of the cyclic nucleotide by the action of cyclic nucleotide phosphodiesterase. With irradiation times of 4-5 sec, the recovery of cGMP was high, probably reflecting a preferential inactivation of cyclic nucleotide phosphodiesterase relative to guanylate cyclase. Exposure times of longer than 6 sec produced consistent cGMP concentrations of approximately 2 pmol/mg brain protein in control animals, indicating effective inactivation of both cyclic nucleotide phosphodiesterase and guanylate cyclase. In the experiments reported here, we have used a microwave exposure time of 7 sec. After killing by microwave irradiation, the animals were decapitated and the brains were removed. In some cases, the brains were dissected into forebrain and hindbrain regions (9). The tissues were homogenized in H_2O (total volume, 6 ml) and samples (0.6 ml) were removed for protein determinations (10). [^{3}H]-cGMP (approximately 2500 cpm) was added to the remainder of the homogenate to monitor recovery. The samples were then adjusted to 0.2N HCOOH and boiled for 5 min. After centrifugation, the supernatant solutions were lyophilized. The dried samples were dissolved in 4.5 ml H_2O and assayed for cGMP using a Schwarz-Mann radioimmunoassay kit (11, 12). The data were analyzed statistically by Student's t test (13).

RESULTS

Cyclo (His-Pro) Elevates the Level of cGMP in Brain: When rats were sacrificed by microwave irradiation for 7 sec, the level of cGMP in brain was approximately 2 pmol/mg protein (Table 1). Administration (IP) of TRH (50 μmol/kg) to rats produced a transient increase in the level of the cyclic nucleotide (Fig. 1). There was a significant increase in concentration when the animals were sacrificed 4 min after injection of the peptide. The highest concentrations of cGMP were observed between 5-15 minutes after TRH injection. By 60 min, the cGMP concentration had returned to the control level. Intraperitoneal injection of cyclo(His-Pro) (50

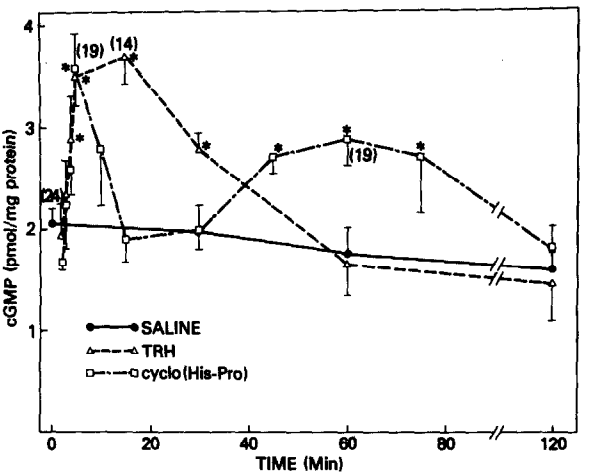

Fig. 1 Time course of the effect of TRH or cyclo(His-Pro) on levels of cGMP in brain. Rats were injected intraperitoneally with solutions (5 mM in saline) of TRH or cyclo(His-Pro) at a dose of 50 μmol/kg. Control animals received saline alone. At the indicated times, animals were sacrificed by microwave irradiation. The brains were then removed. In some cases, the brains were divided into forebrain and hindbrain regions and processed separately. The samples were homogenized and assayed for cGMP content as described in Methods. The points are mean values for groups of 4-5 animals; where more animals were used, their number is indicated in parentheses. The bars indicate SEM. Asterisks indicate values which are significantly different from saline-treated controls at zero time ($p<0.01$).

μmol/kg) also produced an increase in the brain cGMP concentration, but with a somewhat different pattern (Fig. 1). The rates of increase due to TRH or cyclo (His-Pro) were similar. However, cGMP levels did not remain elevated for as long a period of time after cyclo(His-Pro) injection as they did after TRH injection. The maximum elevation of the level of cGMP after cyclo(His-Pro) treatment was observed at 5 minutes and the level returned to baseline by 15 min. An additional effect seen with cyclo(His-Pro) but not TRH was a second transient increase in cGMP concentration with a maximum response at 60 minutes.

An examination was made of the dose-response profiles for the TRH or cyclo(His-Pro) dependent elevations of cGMP concentration at the times characteristic of the peak responses (TRH at 15 min; cyclo(His-Pro) at 5 and 60 min). Elevation of cGMP titers at 15 minutes after intraperitoneal injection of TRH was observed with a dose of 5 μmol/kg of this peptide, but the effect was maximal at 10 μmol/kg (Fig. 2, left panel). Regional dissection indicated that, at this dose of TRH, the elevation of cGMP concentration was primarily in the hindbrain (Fig. 2, right panel). The dose-response profile for changes in levels of cGMP in whole brain at 5 minutes after cyclo(His-Pro) injection (Fig. 2, left panel) indicated that the concentration of the cyclic nucleotide increased with increasing peptide concentration between 5-50 μmol/kg. However, examination of the hindbrain

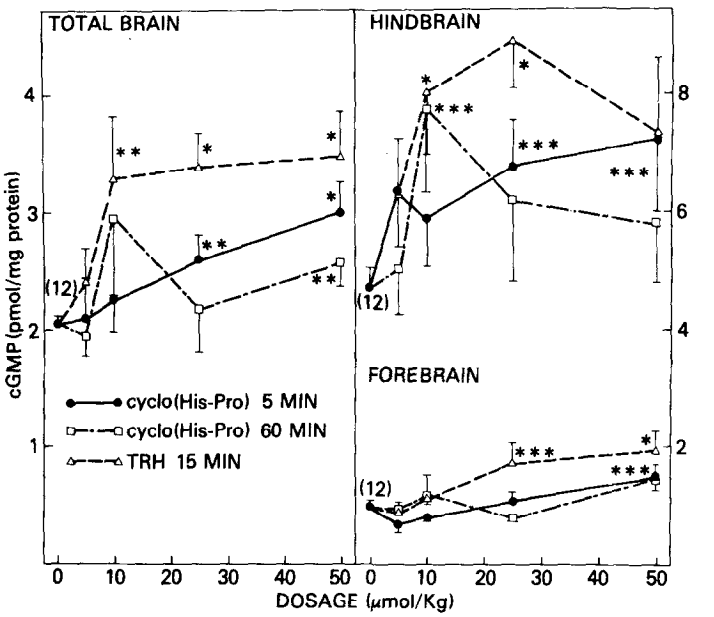

Fig. 2 Effect of concentration of TRH and cyclo(His-Pro) on cGMP levels in total brain, hindbrain, and forebrain. Rats were injected with the indicated dosages of TRH or cyclo(His-Pro) in saline (10 ml/kg). Control animals were injected with saline alone and sacrificed immediately. Animals injected with cyclo(His-Pro) were sacrificed by microwave irradiation at 5 or 60 min; those injected with TRH were sacrificed at 15 min. The brains were removed, separated into forebrain and hindbrain regions and processed for cGMP determination (see Methods). The data for total brain cGMP (left panel) was calculated from the sums of the amounts of cGMP and protein in the forebrain and hindbrain regions (right panel). The points are mean values obtained for groups of 6-9 animals; where more animals were used, their number is indicated in parentheses. The bars indicate SEM. Asterisks indicate values which are significantly different from saline controls (*, $p<0.01$; **, $p<0.02$); and ***, $p<0.05$).

region (Fig. 2, right panel) indicated that 5 μmol/kg of cyclo(His-Pro) produced a nearly maximal effect. Higher concentrations of cyclo(His-Pro) appeared to produce an increase in cyclic nucleotide concentration primarily in the forebrain. The elevation of cGMP concentration at 60 minutes after injection of cyclo(His-Pro) required 10 μmol/kg of the peptide and was observed primarily in the hindbrain (Fig. 2, left and right panels).

Cyclo(His-Pro) Antagonizes the Ethanol-Induced Decrease in Brain cGMP Concentration

Ethanol produced a dose-dependent increase in rat brain cGMP levels (1-3); a dose of 1.5 g/kg elicited an approximately 50% decrease in cyclic nucleotide concentration (Fig. 3A). TRH or cyclo(His-Pro) at a dose of 50 μmol/kg elevated the cGMP concentration in brain in the absence of EtOH (Fig. 1, 3A). The peptides also had the capability of antagonizing the EtOH-induced decrease in brain cGMP levels (Fig. 3A). Rats were injected with the indicated dose of EtOH and then

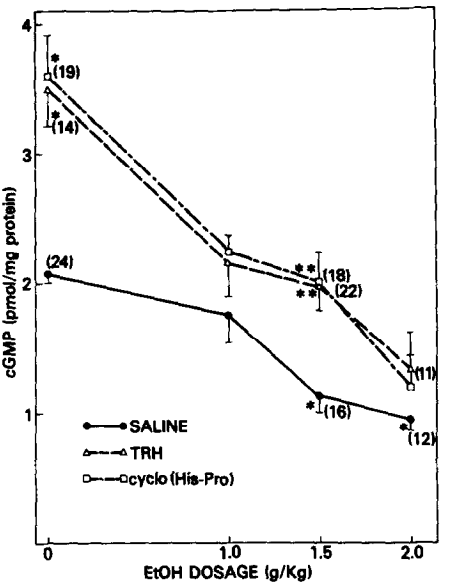

Fig. 3A Effect of TRH or cyclo(His-Pro) on cGMP levels in brain in the presence of various doses of ethanol. Rats were injected intraperitoneally with the indicated dosage of EtOH (4 ml/kg). Five min later, saline, TRH or cyclo(His-Pro) (10 ml/kg; 50 µmol/kg) were injected intraperitoneally. After an additional five min, the animals were sacrificed. Brains were removed and processed for cGMP and protein (see Methods). The points are mean values of 4-8 animals; where more animals were used, their number is in parentheses. Statistical analysis was as in Fig. 1. *, significantly different ($p<0.01$) from saline controls at a dosage of EtOH of 1.5 g/kg.

5 min later, they were injected with saline, TRH or cyclo(His-Pro). Ten min after the EtOH injection, the animals were sacrificed and the brains processed for cGMP determinations. A comparison of the EtOH dose-response curves in the absence or presence of the peptides indicated that the elevated levels of cGMP characteristic of the peptides were more sensitive to decrease by EtOH than was the level in the saline-treated controls. At an EtOH concentration of 1.5 g/kg, TRH or cyclo(His-Pro) completely reversed the effect of EtOH on cGMP levels. However, when the EtOH concentration was increased to 2.0 g/kg, TRH or cyclo(His-Pro) were less effective in reversing the EtOH-dependent decrease in brain cGMP concentration.

The time-course of the effect of EtOH in the absence or the presence of TRH of cyclo(His-Pro) was studied (Fig. 3B). Peptides, where used, were injected 5 min after EtOH. The maximum decrease in brain cGMP concentration was observed 15 min after EtOH injection. When the animals also received injections of TRH or cyclo(His-Pro), the effect of EtOH observed at 5 min after peptide injection was prevented, but EtOH was still effective at 10 min after peptide injection (Fig. 3B). The study presented in Fig. 1 indicated that cyclo(His-Pro) produced an elevation

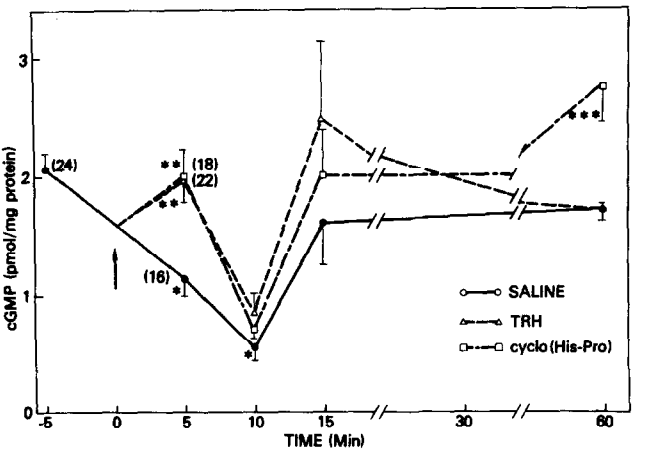

Fig. 3B Time course of the effect of TRH or cyclo(His-Pro) on cGMP levels in brain in the presence of EtOH. Rats were injected intraperitoneally with EtOH solution (4 ml/kg) at a dosage of 1.5 g/kg. Five min later, (see arrow in Figure) saline, TRH or cyclo(His-Pro) was injected at a dosage of 50 µmol/kg (see Fig. 1). At the indicated times, animals were sacrificed. Brains were removed and processed for cGMP and protein as described in Methods. The points are mean values for 3-9 animals; where more animals were used, their number is indicated in parentheses. Statistical analysis was as in Fig. 1. *, significantly different ($p<0.01$) from saline control in the absence of EtOH. ** and ***, significantly different ($p<0.01$ and $p<0.05$ respectively) from saline controls at the indicated times.

of brain cGMP concentration at 60 min which was not observed after injection of TRH. This effect was also observed in the presence of EtOH (Fig. 3B).

A study of the concentration of peptide required to reverse the EtOH-dependent lowering of the cyclic nucleotide concentration is shown in Fig. 4. Significant antagonism was observed with 5 µmol/kg of cyclo(His-Pro) but required at least 10 µmol/kg of TRH.

DISCUSSION

It has previously been shown that TRH elevates brain cGMP titers. The major finding in the present study is that the TRH metabolite cyclo(His-Pro) also elevates the concentration of the cyclic nucleotide in brain (Figs. 1 and 2). There are some fundamental differences in the properties of the two peptides in regulating cGMP levels. At high doses (50 µmol/kg) of the peptides, the peak responses are of similar magnitude (Fig. 1). However, TRH produces a longer-lasting effect. Injection of cyclo(His-Pro) is also characterized by a second transient increase in cGMP concentration which is not observed with TRH. It is noteworthy that the change of cGMP concentration observed at 60 min requires a higher concentration of cyclo(His-Pro) than does the effect seen at earlier times (Fig. 2, right panel). It is unlikely that the effect on cGMP concentration elicited by cyclo(His-Pro)

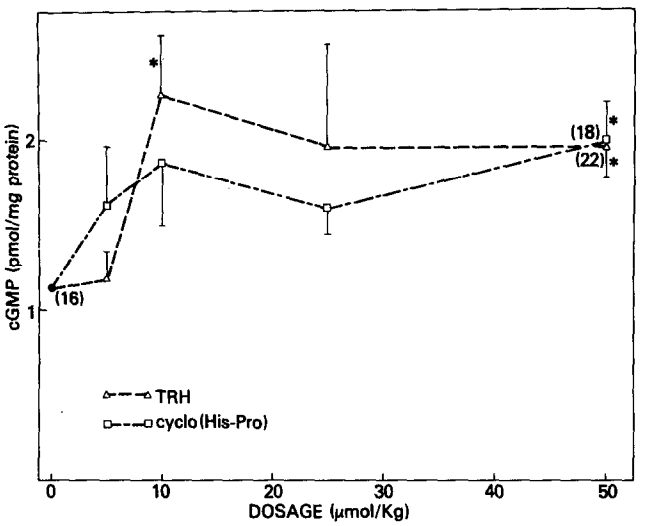

Fig. 4 Effect of concentration of TRH and cyclo(His-Pro) on cGMP levels in the presence of EtOH. Rats were injected intraperitoneally with EtOH at a dosage of 1.5 g/kg; five min later, the animals were injected intraperitoneally with saline or the indicated dosages of TRH or cyclo(His-Pro) in saline (10 ml/kg). After an additional 5 min, the animals were sacrificed. Brains were removed and processed for cGMP and protein determinations as described in Methods. The points are mean values for 4-9 animals; where more animals were used, their number is indicated in parentheses. Statistical analysis was as described in Methods. *, significantly different ($p<0.01$) from saline controls in the presence of EtOH.

at 60 min is correlated with the antagonism of EtOH-induced changes in cyclic nucleotide levels, since such an effect is not elicited by TRH. It therefore appears that cyclo(His-Pro) may have some effects on cyclic nucleotides that are related to other brain functions.

The dose-response properties of the peptide in elevating cGMP levels in hindbrain (Fig. 2) suggest that the maximum increase in cGMP concentration is greater with TRH than with cyclo(His-Pro); however, the concentration of cyclo(His-Pro) required to generate the maximum effect (5 μmol/kg) is somewhat less than that required for TRH (10-25 μmol/kg). These data indicate that the effect of TRH on brain cGMP levels might be the result of the conversion of the peptide to cyclo(His-Pro). However, in the absence of studies whereby TRH is prevented from being metabolized to cyclo(His-Pro), it is impossible to evaluate the alternative model that both TRH and cyclo(His-Pro) can increase the concentration of cGMP in brain. In any event, the observation that cyclo(His-Pro) is an active agent in regulating cGMP levels should be taken into consideration in evaluating the effects of TRH.

Ethanol at doses between 1-2 g/kg, effectively lowers the concentration of cGMP in brain. As shown in Fig. 3A, high doses of either TRH or cyclo(His-Pro)

effectively antagonize the EtOH-dependent decrease in the brain cGMP level. Cyclo(His-Pro) appears to be somewhat more potent than TRH in antagonism of the effect of EtOH (Fig. 4); 5 μmol/kg of cyclo(His-Pro) appears to reverse the effect of EtOH while a similar dose of TRH has no effect. However, at higher doses, TRH produces a greater elevation of cGMP concentration, an effect similar to that seen in the absence of EtOH (Fig. 2). Thus, it appears that while cyclo(His-Pro) may exercise its effect at lower doses than TRH, TRH tends to produce a greater response at intermediate doses (between 10-25 μmol/kg).

Studies in this laboratory have documented the in vivo conversion of TRH to cyclo(His-Pro) and described the properties of enzymes involved in the metabolism of TRH (6,7,14). There is accumulating evidence that cyclo(His-Pro) plays an important role in cerebral function. Cyclo(His-Pro) is more potent than TRH in reversing EtOH-dependent narcosis in rats (7). In these animals, the diketopiperazine also produces a hypothermia which is antagonized by TRH (8). The cyclic dipeptide inhibits prolactin secretion and antagonizes the TRH-dependent stimulation of prolactin secretion (15). Future studies will probably reveal other biological properties of this unique cyclic dipeptide.

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