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Effects of Intra-Amygdalar Thyrotropin Releasing Hormone (TRH) and Its Antagonism by Atropine and Benzodiazepines During Stress Ulcer Formation in Rats

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RAY, A., P. G. HENKE AND R. M. SULLIVAN. *Effects of intra-amygdalar thyrotropin releasing hormone (TRH) and its antagonism by atropine and benzodiazepines during stress ulcer formation in rats.* PHARMACOL BIOCHEM BEHAV **36**(3) 597–601, 1990.—Bilateral intra-amygdalar (i/am) microinjections of TRH (1 and 10 μg) and physostigmine (10 μg) into the central nucleus (CEA) aggravated cold restraint stress (3 hr at 4°C) induced gastric ulcer formation in rats, whereas atropine (1, 5 and 10 μg) attenuated this phenomenon. Similar stress ulcer reducing effects were seen with chlordiazepoxide (CDP, 10 mg/kg, IP) and midazolam (1, 3 and 10 μg, i/am). Pretreatment of rats with atropine or CDP antagonized the ulcerogenic effects of both TRH and physostigmine. Further, when administered intra-CEA, midazolam neutralized the effects of TRH in a dose-related manner. These results are discussed in light of TRH-acetylcholine-benzodiazepine/GABA interactions within the amygdaloid complex during stress ulcer formation.

Thyrotropin releasing hormone Acetylcholine Benzodiazepines Central amygdala Stress ulcers

THE amygdaloid complex is important for the organism's response to stress. The central amygdalar nucleus (CEA), through its connections with the hypothalamus and/or brain stem areas, is particularly crucial for the maintenance of gastric mucosal integrity during exposure to experimental stressors (8, 11, 14). For example, (a) lesions in the CEA attenuated restraint stress gastric pathology, whereas low level electrical stimulations of the amygdala induced gastric lesions only when localized in the CEA, and (b) immobilization stress altered multiple unit activity in the CEA and a correlation between such neural activity, emotionality and stress ulcer susceptibility was suggested. Neuropharmacological data also support the proposed involvement of CEA during stress. Intra-CEA microapplications of several neurotransmitters (like some biogenic amines, amino acids and neuropeptides), and their modulators, influence stress ulcer formation in a rather complex manner (27–29).

Thyrotropin releasing hormone (TRH), a tripeptide, is widely distributed in the central nervous system (CNS) and evidence indicates that it may act as a neurotransmitter/neuromodulator. Several reports have shown its probable physiological role in the CNS and complex pharmacological actions have been defined (17). Specific binding sites or receptors for TRH are now identified in the dorsal vagal complex, hypothalamus and amygdala, areas that are seemingly crucial in the regulation of gastric mucosal integrity during stress (7, 9, 17). Recent data have also shown that both intraventricular as well as intra-CEA injections of TRH induced gastric lesions and also aggravated the gastric mucosal response to restraint stress (13,15), indicating that the CEA may in fact be one of the neuroanatomical substrates for this effect of the neuropeptide. Interactions of TRH with other neurotransmitter systems are also known and a recent study showed that neurotensin and dopamine interact with TRH in the CEA during stress ulcer formation (2, 13, 17). Cholinergic pathways have been implicated in some TRH effects and peripheral muscarinic mechanisms are seemingly involved in a gastric ulcerogenic effects of this neuropeptide (13, 23, 26, 38, 39). However, the role of central cholinergic neurons, particularly those in the amygdaloid complex, and their regulatory influence on TRH effects on gastric mucosal integrity during stress, is still not clearly defined.

Benzodiazepines (BZD) are recognized as antistress/antianxiety agents and both peripheral and central administration of BZD's attenuate stress ulcer formation (12,34). The CEA is also sug-

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gested to be a possible substrate for the anxiolytic effects of BZD's, and the GABA-BZD receptor complex in this area is seemingly important for their stress ulcer modulating effects (33,34). In addition to binding with the GABA-BZD receptor complex, BZD's also compete with TRH for the latter's binding sites in the CNS including those in the amygdaloid complex (30–32). Further, BZD's are also known to modulate cholinergic mechanisms during stress (19). The physiological significance of such interactions, however, remains to be determined.

In view of the fact that the amygdaloid complex is rich in TRH receptors, cholinergic neuronal elements and BZD binding sites, we investigated the formation of gastric mucosal lesions as a function of the interactions of TRH with (a) acetylcholine (ACh), and (b) BZD-GABA mechanisms in the CEA during immobilization stress in rats.

METHOD

Male Wistar rats, 90–120 days old, were used. They were housed individually, and maintained at a temperature of 22 ± 2°C and in a 12-hr light–12-hr dark cycle (lights on at 8 a.m.) with free access to food and water. For surgery, rats were anesthetised with sodium pentobarbital (50 mg/kg, IP) and secured in a stereotaxic apparatus. Twenty-three gauge stainless steel guide cannulae (Plastic Products) were lowered bilaterally into the central amygdalar nucleus using the following coordinates: 2.0 P (to bregma), 4.1 L (to midline) and 7.5 V (to dura), skull horizontal. The cannulae were secured in position by stainless steel screws and dental acrylic. The rats were allowed a one-week postoperative recovery period and then deprived of food (but not water), 24 hr prior to restraint. Intra-CEA (i/am) saline (0.9%) or drug injections were made bilaterally in conscious, hand-held animals, in a volume of 2 μl, slowly over a period of 1 min with a 5-μl Hamilton Syringe. The injection cannula (30 gauge stainless steel), attached to the syringe with 50 cm of microbore tubing, was allowed to remain in the guide cannula for an additional 3-min period to allow diffusion of the injection solution into the brain. Immediately after i/am injections, the rats were immobilized in Plexiglas restrainers (Fisher Scientific) at 4°C. After 3 hr of cold restraint the rats were sacrificed with an overdose of sodium pentobarbital (IP). The stomachs were then dissected out, cut open along the greater curvature, washed in cold water and examined microscopically (×10) for gastric mucosal lesions. The number and severity (cumulative length in mm, to the nearest 0.1 mm) of such lesions were determined. Following removal of stomachs, the rats received intracardiac perfusions of 0.9% saline followed by 10% formalin. The brains were then extracted from the skull, embedded in paraffin, sectioned at 15 μ and stained in thionin.

The drugs used were thyrotropin releasing hormone (TRH), physostigmine salicylate, atropine sulfate, atropine methyl nitrate (all from Sigma Chemical Co., St. Louis, MO) and chlordiazepoxide hydrochloride and midazolam maleate (both from Hoffmann-La Roche, Canada). All drugs were dissolved in physiological saline and injected i/am, except chlordiazepoxide and atropine methyl nitrate, which were injected IP. In the interaction studies, drug pretreatment times were 30 min (for IP) and 15 min (for i/am) respectively.

The results were analysed using the Kruskal-Wallis one-way ANOVA for nonparametric data, followed by the Mann-Whitney U-test, two-tailed, for subsequent multiple comparisons between groups. A p value of at least 0.05 was used as the level of significance in all statistical tests.

RESULTS

Effects of TRH, Atropine, Physostigmine and CDP on Stress Ulcers

The analysis of the gastric pathology data revealed that both the mean number of ulcers and the mean cumulative ulcer length, per rat, were significantly different across all groups [$H(12) = 61$, $p<0.001$; and $H(12) = 59$, $p<0.001$, respectively, Kruskal-Wallis test]. Specifically, intra-CEA TRH (1 and 10 μg) aggravated stress ulcer formation in a dose-related manner. As shown in Table 1, significant differences from control values were seen in both ulcer number and severity for TRH (10 μg). The anticholinergic agent, atropine (1, 5 and 10 μg/am) attenuated stress gastric lesions, also in a dose-dependent manner, when injected bilaterally into the CEA. The anticholinesterase agent, physostigmine (10 μg/am), on the other hand, potentiated the severity of stress ulcers, and though a 28% increase was observed in the ulcer frequency data, this was not statistically significant ($p>0.05$). Pretreatment of rats with atropine clearly prevented and even reversed the facilitatory effects of physostigmine as well as TRH on gastric mucosa during stress. Further, similar neutralization of the ulcerogenic effect of physostigmine was also seen with atropine methylnitrate (1 mg/kg) pretreatment. Chlordiazepoxide (CDP, 10 mg/kg, IP), as expected, reduced both the frequency and severity of stress ulcers, compared to control values. In addition, CDP pretreatment was also seen to reverse the effects of i/am TRH or physostigmine.

TABLE 1

EFFECTS OF INTRA-AMYGDALAR (i/am) APPLICATIONS OF TRH, ATROPINE (A), PHYSOSTIGMINE (PHY) AND IP CHLORDIAZEPOXIDE (CDP) ON STRESS ULCER FORMATION

Treatment (μg i/am)	n	Mean Gastric Pathology (± S.D.) Ulcer Number	Ulcer Severity
Controls	8	6.3 ± 0.7	1.8 ± 0.5
TRH (1)	7	8.7 ± 1.4†	2.6 ± 0.6
TRH (10)	8	13.8 ± 4.2*	6.4 ± 2.2*
A (1)	6	4.7 ± 1.1‡	1.9 ± 1.8
A (5)	6	2.8 ± 0.9*	0.6 ± 0.2*
A (10)	6	1.8 ± 1.1*	0.4 ± 0.3*
PHY (10)	7	8.1 ± 3.2	3.8 ± 2.3‡
AMN (1 mg) + PHY	6	3.2 ± 0.9*§	0.6 ± 0.1*§
A (10) + PHY	6	3.3 ± 1.6†¶	0.9 ± 0.7¶
A (10) + TRH (10)	6	4.1 ± 1.9‡#	0.7 ± 0.3*#
CDP (10 mg/kg)	6	2.6 ± 1.1*	0.6 ± 0.2*
CDP + TRH	6	4.8 ± 1.3‡#	1.2 ± 0.4‡#
CDP + PHY	6	4.0 ± 1.4†¶	0.9 ± 0.4†¶

*$p<0.002$; †$p<0.02$; ‡$p<0.05$ (compared to controls).
§$p<0.002$; ¶$p<0.02$ (compared to PHY group).
#$p<0.002$ [compared to TRH (10) group].
AMN: Atropine methyl nitrate.

TRH-Midazolam Interactions During Stress Ulcer Formation

Overall nonparametric ANOVA for the data showed that the mean ulcer number and ulcer severity, per rat, were significantly different across all groups [$H(7) = 25$, $p<0.001$; and $H(7) = 27$, $p<0.001$, respectively, Kruskal-Wallis test]. As seen in the earlier experiment, TRH (10 μg) aggravated stress ulcer formation (Table 2). The BZD, midazolam (3 and 10 μg/am) produced dose-dependent reductions in gastric stress ulcerogenesis. A facilitatory trend was, however, seen with the lowest dose (1 μg) of this drug, but this enhancement in the mucosal response to stress was not significantly different from controls ($p>0.05$). Further, midazo-

TABLE 2

INTERACTIONS OF TRH WITH MIDAZOLAM (MIDAZ) AFTER INTRA-AMYGDALAR (i/am) APPLICATIONS DURING STRESS ULCER FORMATION

Treatment (μg i/am)	n	Mean Gastric Pathology (±S.D.) Ulcer Number	Ulcer Severity
Controls	8	6.6 ± 1.7	2.1 ± 1.7
TRH (10)	6	10.6 ± 2.5*	4.8 ± 1.5*
Midaz (1)	7	7.3 ± 1.6	2.7 ± 0.9
Midaz (3)	7	3.5 ± 0.9*	0.9 ± 0.2*
Midaz (10)	7	1.3 ± 1.0*	0.3 ± 0.2*
Midaz (3) + TRH	6	9.0 ± 2.1	3.3 ± 1.5‡
Midaz (10) + TRH	8	5.4 ± 1.5†	1.4 ± 0.6†
TRH + Midaz (10)	6	7.5 ± 1.9†	3.0 ± 1.3‡

*$p<0.002$ (compared to controls).
†$p<0.002$; ‡$p<0.05$ (compared to TRH group).

lam pretreatment (3 and 10 μg) dose-dependently attenuated the effects of TRH (10 μg), when both were injected intra-CEA, at 15-min intervals. When the order of treatment was reversed, both TRH and midazolam tended to cancel each other's effects on the gastric mucosa during stress.

Histological examination revealed that the majority of the implanted cannula tips were located in the dorsal region of the CEA or immediately dorsal to this structure. When the cannula tips were outside of or unilaterally in the CEA or destroyed parts of it, these data were not included in the statistical analysis. In the CEA, injection sites were located in both lateral and medial parts of this area. There were, however, no differential effects in the gastric pathology data observed in these cases. On the other hand, in rats with cannula tips outside of or unilaterally in the CEA, the gastric pathology data after different drug treatments were more or less similar to that of the i/am saline control group.

DISCUSSION

Neuropeptides regulate the ulcerogenic response to stress and central mechanisms are involved in the effects of TRH on gastrointestinal function and ulceration (36,37). The CEA is a probable substrate for some of these effects (13, 15, 16, 23), and our present data reaffirms that TRH is clearly one of the facilitatory mediators at the level of the CEA during stress ulcer formation. However, possible diffusion to other amygdaloid areas after (2 μl) intra-CEA injections cannot be ruled out. But the current findings, as well as earlier reports indicate that rats with cannula tips outside of the CEA had similar ulcer pathology in both drug-treated or vehicle-injected groups. Furthermore, a number of earlier studies have also shown that the CEA is the crucial amygdalar area during stress ulcerogenesis (14, 27–29).

Central cholinergic pathways are facilitatory for stress responses (3, 4, 19). The amygdaloid complex is rich in cholinergic neural elements and behavioral data have shown the significance of ACh in this limbic area (6, 18, 20). Further, studies have shown that some amygdaloid nuclei are closely associated with parasympathetic activity and the regulation of gastric secretions. According to another report, the centromedial amygdalar region, via its connections with vagal nuclei, also influence gastric functions (8,22). Though earlier studies have shown peripheral and central mechanisms for ACh during gastric stress ulcer formation (10,15), the exact locus of this effect is not clearly defined. Our findings show that (a) i/am atropine inhibited stress ulcers, (b) physostigmine aggravated this phenomenon, and (c) atropine pretreatment prevented physostigmine effects, both i/am—all indicating a facilitatory cholinergic (muscarinic) mechanism, at the level of the CEA, during stress ulcer formation. In addition, the peripherally acting anticholinergic agent, atropine methylnitrate, also prevented the effects of physostigmine on the gastric mucosa. Taken together, these findings indicate the involvement of the cholinergic brain-gut axis and the role of the CEA as one of the substrates for the mediation of the effects of the cholinomodulators.

Interactions involving cholinergic neurons and TRH are reported throughout the neuraxis (39). For example, TRH is known to enhance the sensitivity of muscarinic receptors to ACh and also the synthesis and release of this transmitter. Some central effects of TRH are also known to be mediated via activation of cholinergic pathways (26,38). A recent study showed that presynaptic TRH terminals may modulate vagal activity at the level of the dorsal motor nucleus of the vagus in the brain stem and in turn contribute to the acid hypersecretion and experimental ulcer formation in the stomach (16). Earlier data indicated the role of peripheral muscarinic mechanisms in the ulcerogenic effects of TRH (13) and our present finding that atropine blocked/reversed TRH effects (both i/am) on the gastric mucosa shows the importance of intra-CEA cholinergic mechanisms in the stress ulcer aggravating effects of this neuropeptide. During stress TRH probably also activates cholinergic neurons in the CEA, which in turn produces the observed disruptive changes on gastric mucosa.

Several studies have shown that BZD's attenuate some behavioral and endocrinal responses to stress and the amygdaloid complex (and the CEA) has been implicated as a substrate for the antistress/antianxiety effects of these agents (12, 24, 33, 34). The BZD-GABA receptor complex in the CEA is seemingly important for the protective effects of CDP on the gastric mucosa (34) and our finding that midazolam dose-dependently attenuated stress ulcer formation after intra-CEA applications is consistent with the proposed role of this limbic area (and its BZD-GABA receptor complex) in the regulation of this visceral response to the stressor. Interestingly, the CEA was shown to be extremely sensitive to midazolam (30), a reason why this drug was selected for this study. Enhancement of GABA-ergic transmission is one of the mechanisms which BZD's use to elicit their pharmacological effects, and both BZD receptors as well as GABA are found in the CEA (1,40). Further, intra-CEA microapplications of GABA also reduced the gastric ulcerogenic effects of stress (34). In addition to acting through GABA, BZD's also reportedly combine and compete, rather specifically, with TRH binding sites in different brain areas, including those in the amygdala (30–32). Though these in vitro binding studies are supported to some extent by behavioral data (21), no clearcut relationship was observed between TRH displacing ability and antistress/antianxiety effects for these drugs. In view of the proposed nature of involvement of TRH (facilitatory) and BZD-GABA (inhibitory) during stress, interactions between these two modulators are possible at the level of the CEA during stress ulcer formation. Our studies show that both CDP (IP) and midazolam (i/am) prevented/reversed the i/am TRH effects on the gastric mucosa, indicating that the CEA is a probable site for TRH-BZD-GABA interactions during immobilization stress. Taken together, it is reasonable to speculate that, in the CEA, BZD's (a) combine with the BZD-GABA receptor complex and modulate GABA, and/or (b) compete with TRH binding sites or receptors. Whatever the pharmacodynamics involved, in either case, the net effect is an attenuation of the stress response on the gastric mucosa.

Interestingly, CDP (10 mg/kg, IP) also antagonized the effects of intra-CEA physostigmine. The importance of the amygdaloid

complex (and the CEA) in the mediation of BZD and ACh effects have been shown and interactions between these two agents are reported during aversive experiences like stress (19). Further, a centrally mediated vagolytic effect for BZD's has also been proposed (5). Consistent with this, recent data showed that interference with central GABA-ergic transmission mimicked stress-induced changes in parasympathetic function and forebrain GABA, in particular, clearly controls central vagal outflow to the stomach through the dorsal motor nucleus (25). In view of the above, it is not surprising that the antistress effects of CDP presumably prevented the disruptive effects of physostigmine (and thereby ACh) on the gastric mucosa. In doing so, CDP may have blocked (a) TRH, and (b) TRH-activated ACh neural systems.

In conclusion, complex interactions take place at the level of the CEA between TRH, cholinergic and BZD-GABA mechanisms during stress ulcer formation. Whereas TRH and ACh tend to facilitate this stress response, BZD's attenuate this phenomenon as well as the effects of TRH and ACh. These neuropharmacological data further confirm to role of the CEA in the regulation of gastric stress ulcerogenesis.

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