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Involvement of glutamate and γ-aminobutyric acid (GABA)-ergic systems in thyrotropin-releasing hormone-induced rat cerebellar cGMP formation

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Abstract

The increase in cyclic guanosine 3′,5′-monophosphate (cGMP) caused by subcutaneous injection of thyrotropin-releasing hormone (TRH) tartrate was observed in a region-specific manner in the rat cerebellum. TRH tartrate (TRH-T) (2.8, 7.0 and 17 mg/kg as free TRH, s.c.) produced dose-dependent increases in cGMP levels markedly in the cerebellar superior and inferior vermis, and a smaller but still significant increase in the cerebellar hemispheres and brainstem but no significant increases in other brain regions. The TRH-induced increase in the cGMP level in the cerebellum was suppressed by pretreatment with muscimol, THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3(2*H*)-one) or MK-801 (dizocilpine maleate) and partially suppressed by atropine but was not suppressed by chlordiazepoxide, oxazepam, phentolamine, propranolol, cyproheptadine, haloperidol, baclofen or DNQX (6,7-dinitroquinoxaline-2,3-dione), suggesting the possible involvement of GABA (γ-aminobutyric acid)(A)-ergic, *N*-methyl-D-aspartate (NMDA)-type glutamatergic and cholinergic systems. These results suggest that excitatory amino acids may be involved in the cGMP formation caused by TRH in the cerebellar areas, and that cGMP formation is inhibited by enhancement of $GABA_A$ receptor function.

Keywords: TRH tartrate (thyrotropin-releasing hormone tartrate); cGMP; Cerebellum; GABA (γ-aminobutyric acid); MK-801; Purkinje cell

1. Introduction

Thyrotropin-releasing hormone (TRH) is a hypothalamic tripeptide (L-pyroglutamyl-L-hystidyl-L-proline amide) neurohormone which stimulates the release and synthesis of thyrotropin from the anterior pituitary via the hypophyseal portal system. In addition, TRH is known to have profound pharmacological effects on the central nervous system (e.g., modulation of the monoaminergic system, enhancement of learning and memory functions, antagonism of hypnotic, sedative and hypothermic states (Burgus et al., 1970; Lechan et al., 1986; Sharif, 1985), etc.).

While TRH has been reported to ameliorate the ataxic condition in several mutant mice with neuropathological changes primarily in the cerebellum (Matsui et al., 1983; Ando and Matsui, 1986), the mechanism underlying the ameliorating effect of TRH remains unclear. The report by Redos et al. (1976) that ethanol decreases cerebellar cyclic guanosine 3′,5′-monophosphate (cGMP), and the correlation of this change with ataxic behavior, led us to investigate the mechanism by which TRH induces cGMP production in various cerebellar areas. Although Mailman et al. (1979) reported that the time-course of TRH immunoreactivity in cerebellar homogenates roughly paralleled the time-course of cGMP elevation in the cerebellum after the administration of TRH and that the TRH-induced elevation in cerebellar cGMP was not affected by cerebellar climbing fiber lesions caused by 3-acetylpyridine and also not blocked by haloperidol which is known to lower the cGMP content by decreasing the activity of mossy fibers, they did not clarify the mechanism by which TRH acts to increase cerebellar cGMP. There are currently several lines of evidence indicating that TRH enhances *N*-methyl-D-aspartate (NMDA)-elicited responses in the cerebral cortex, hippocampus and spinal cord (Kasparov et al., 1994; Stocca

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and Nistri, 1994, 1995; Chizh and Headley, 1994). On the other hand, Cott et al. (Cott and Engel, 1977; Cott et al., 1976) reported that the analeptic activity of TRH or the locomotor stimulation induced by TRH was antagonized by agents which enhance GABA (γ-aminobutyric acid) transmission or the muscarinic receptor antagonist, atropine.

In the present study, therefore, to clarify the mechanism by which TRH stimulates cGMP formation, we investigated the effects of benzodiazepine agonists including chlordiazepoxide which has been suggested to be a pituitary-type TRH receptor antagonist (Sharif et al., 1983; Sharif and Burt, 1984; Simasko and Horita, 1982, 1984), excitatory amino-acid receptor antagonists, GABA receptor agonists and a muscarinic receptor antagonist. Additionally, we examined the possibility of the involvement of monoaminergic neurotransmission.

Some of the results have been presented in abstract form (Nakayama et al., 1995).

2. Materials and methods

2.1. Animals

Male Jcl;Wistar rats weighing 250 to 300 g were used in these experiments. The animals were maintained under controlled environmental conditions (12-h dark/light cycle, 24 ± 1°C and 55 ± 5% relative humidity) and given free access to a standard chow and to water.

2.2. Chemicals

TRH (thyrotropin-releasing hormone) tartrate, DNQX (6,7-dinitroquinoxaline-2,3-dione), chlordiazepoxide and oxazepam were synthesized at Takeda Chemical Industries. Propranolol, atropine, muscimol and baclofen were obtained from WAKO Pure Chemical, Ind., and phentolamine, cyproheptadine and haloperidol were purchased from Sigma Chemical, Ind. MK-801 (dizocilpine maleate) and THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3(2H)-one) hydrochloride were purchased from Funakoshi, Ind. The cGMP assay kit was purchased from Yamasa Shoyu K.K. All other chemicals were of standard reagent grade.

2.3. Time-course of changes in cGMP levels in different cerebellar areas after subcutaneous injection of TRH tartrate

Rats were given a subcutaneous injection of TRH tartrate (25 mg/kg, equivalent to 17 mg/kg as free TRH) and then killed at various times for the determination of cGMP by a radioimmunoassay (RIA) method. TRH tartrate was dissolved in saline. The data were expressed as the means ± S.E. for 5 animals.

2.4. Effects of various compounds on TRH tartrate-induced cGMP formation in different cerebellar areas

Rats were given an intraperitoneal injection of chlordiazepoxide (30 mg/kg), oxazepam (30 mg/kg), phentolamine (10 mg/kg), propranolol (10 mg/kg), DNQX (30 mg/kg), MK-801 (dizocilpine maleate, 0.3, 1 mg/kg), muscimol(1 mg/kg), THIP hydrochloride (16 mg/kg), baclofen (5, 10 mg/kg), haloperidol (2 mg/kg), cyproheptadinne (10 mg/kg) or atropine (50 mg/kg). Thirty minutes later, TRH tartrate (25 mg/kg) was administered subcutaneously. Ten minutes after treatment with TRH tartrate, the animals were killed by microwave irradiation (8 kW for 1.32 s). After killing, the head was cooled in an ice water bath, and the brain was dissected. The cGMP levels in various cerebellar areas (superior and inferior vermis and hemispheres) and the brainstem were determined using a RIA method.

2.5. Cyclic nucleotide assay

After dissection of each brain region, the cerebellum was divided into superior vermis, inferior vermis and hemispheres. Each tissue was homogenized with a homogenizer (polytron, Kinematica, Switzerland) in 10 ml of 6% trichloroacetate per 100 mg of cerebellar tissue. The homogenate was then centrifuged for 15 min at 8000 × g. The pellet was dissolved in 4% NaOH and used for determination of protein content (Lowry's method, Lowry et al., 1951). The supernatant was diluted with water, and trichloroacetate was extracted and removed with diethylether. Treated samples were assayed for cyclic nucleotide content using RIA procedures.

MK-801, muscimol, THIP, phentolamine, propranolol, atropine and TRH tartrate were dissolved in saline, and the other compounds were suspended in a 5% gum arabic solution. The data were expressed as the means ± S.E. for 5 animals.

2.6. Statistics

The significance of differences between each compound-treated group and the control group was evaluated using Student's t-test (effects of compounds other than MK-801 and baclofen) or Student's t-test following analysis of variance (ANOVA) (effects of MK-801 and baclofen), and P values of < 0.05 were considered statistically significant.

3. Results

As shown in Fig. 1, cGMP levels in the cerebellar areas tended to reach a maximum level 10 min after the subcutaneous injection of TRH tartrate (25 mg/kg equivalent to 17 mg/kg as free TRH). Therefore, the cGMP content was

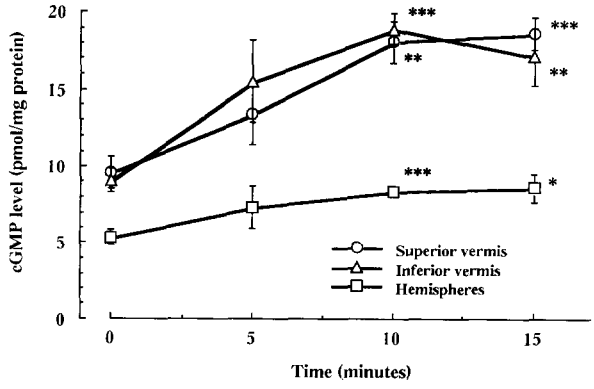

Fig. 1. Time-course of TRH tartrate-induced cGMP formation in the cerebellar areas of the rat brain. TRH tartrate (17 mg/kg as free TRH) was administered subcutaneously (s.c.). cGMP levels in each cerebellar area were determined by a radioimmunoassay method. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with basal levels (Time 0) (Student's t-test).

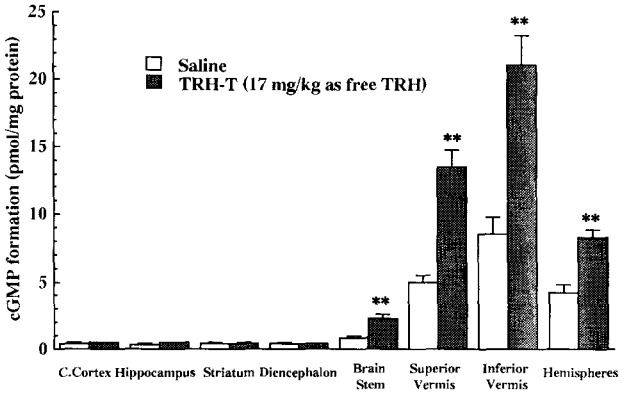

Fig. 2. Effect of TRH tartrate on cGMP formation in various brain regions of rats. Rats were killed by focused microwave irradiation 10 min after injection of TRH tartrate (17 mg/kg, s.c. as free TRH) or saline (SAL), and the brain was dissected into eight areas (cerebral cortex, hippocampus, striatum, diencephalon, brain stem, cerebellar vermis (superior, inferior) and cerebellar hemispheres). After extraction, the cGMP level in each brain region was determined by a radioimmunoassay method. Each column represents the mean ± S.E. for 5 animals. ** $P < 0.01$ compared with saline (Student's t-test).

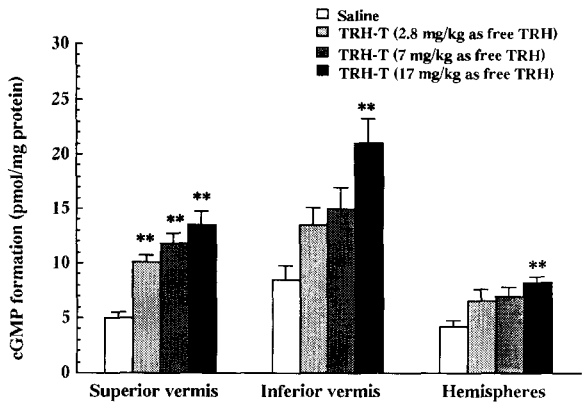

Fig. 3. Effect of TRH tartrate on cGMP formation in the cerebellar areas of the rat brain. Rats were killed by focused microwave irradiation 10 min after injection of TRH tartrate (4, 10 or 25 mg/kg, equivalent to 2.8, 7.0 or 17 mg/kg, s.c. as free TRH) or saline (SAL), and the cGMP level in the cerebellar areas was determined by a radioimmunoassay method. Each column represents the mean ± S.E. for 5 animals. ** $P < 0.01$ compared with saline (Dunnett's test).

determined 10 min after treatment with TRH tartrate in subsequent experiments. A smaller but still significant increase in the cGMP level was observed in the brainstem, although no significant increases were seen in other brain regions such as the cerebral cortex, hippocampus, striatum and diencephalon (Fig. 2). TRH tartrate (4, 10 and 25 mg/kg, equivalent to 2.8, 7.0 and 17 mg/kg as free TRH, respectively) produced dose-dependent increases in cGMP levels in the superior and inferior vermis and hemispheres (Fig. 3).

The TRH tartrate-induced increases in cGMP levels in various cerebellar areas were not suppressed by pretreatment with cholordiazepoxide (benzodiazepine receptor agonist and pituitary-type TRH receptor antagonist) or oxazepam (benzodiazepine receptor agonist) (Fig. 4A, 4B). Likewise, phentolamine (non-selective α-adrenoceptor antagonist) did not suppress the increase in cGMP caused by TRH tartrate (Fig. 5A). Also, propranolol (β-adrenoceptor antagonist) did not have a significant effect on the TRH

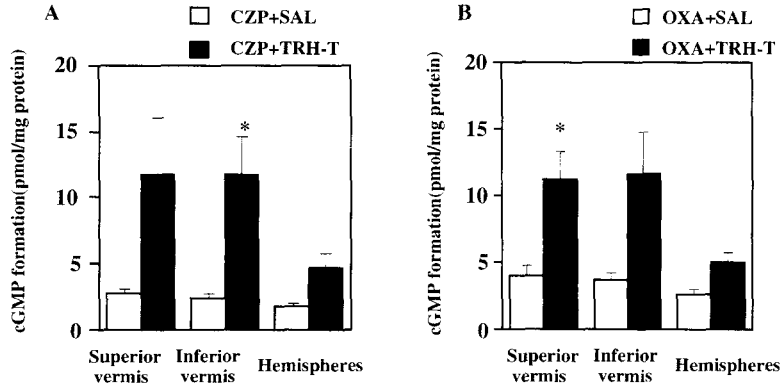

Fig. 4. Effects of benzodiazepine receptor agonists on TRH tartrate-induced cGMP formation in the cerebellar areas of the rat brain. (A) Chlordiazepoxide (CZP, 30 mg/kg), (B) oxazepam (OXA, 30 mg/kg) or saline (SAL) was given intraperitoneally (i.p.) 30 min before injection of TRH tartrate (17 mg/kg, s.c. as free TRH). Rats were killed by focused microwave irradiation 10 min after injection of TRH tartrate or saline (SAL). cGMP levels in each cerebellar area were determined by a radioimmunoassay method. Each column represents the mean ± S.E. for 5 animals. * $P < 0.05$ compared with compounds + SAL (Student's t-test).

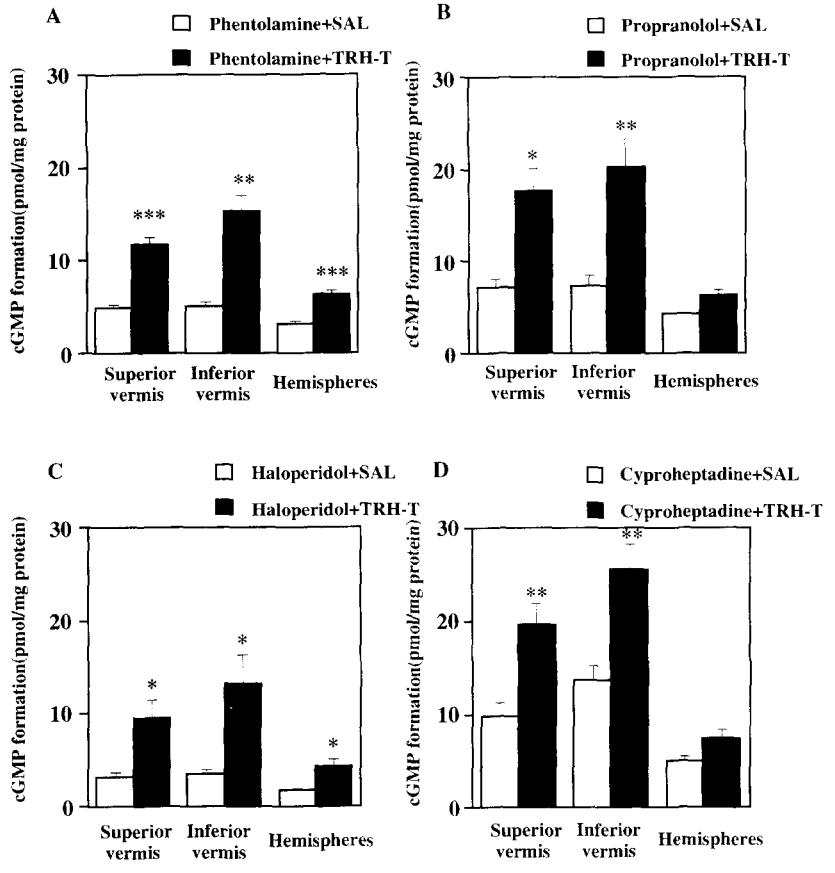

Fig. 5. Effects of monoamine receptor antagonists on TRH tartrate-induced cGMP formation in the cerebellar areas of the rat brain. (A) Phentolamine (30 mg/kg, i.p.), (B) propranolol (30 mg/kg, i.p.), (C) haloperidol (2 mg/kg, i.p.), (D) cyproheptadine (10 mg/kg, i.p.) or saline (SAL) was given 30 min before injection of TRH tartrate (17 mg/kg, s.c. as free TRH). Rats were killed by focused microwave irradiation 10 min after injection of TRH tartrate or saline (SAL). cGMP levels in each cerebellar area were determined by a radioimmunoassay method. Each column represents the mean ± S.E. for 5 animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with compounds + SAL (Student's t-test).

tartrate-induced increase in cGMP levels (Fig. 5B). Haloperidol (dopamine D_2 receptor antagonist) decreased the cGMP levels by reducing the activity of mossy fibers

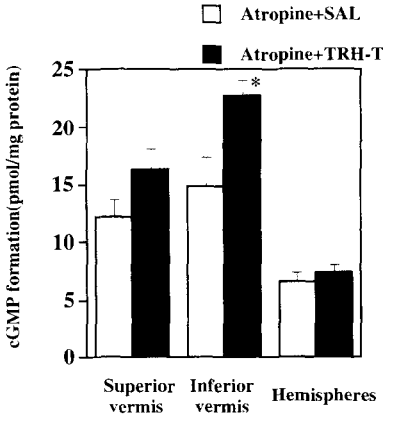

Fig. 6. Effect of a muscarinic receptor antagonist on TRH tartrate-induced cGMP formation in the cerebellar areas of the rat brain. Atropine (50 mg/kg, i.p.) or saline (SAL) was given 30 min before injection of TRH tartrate (17 mg/kg, s.c. as free TRH). Rats were killed by focused microwave irradiation 10 min after injection of TRH tartrate or saline (SAL). cGMP levels in each cerebellar area were determined by a radioimmunoassay method. Each column represents the mean ± S.E. for 5 animals. * $P < 0.05$ compared with Atropine + SAL (Student's t-test).

but did not suppress the TRH tartrate-induced increase in cGMP levels (Fig. 5C). Cyproheptadine (5-HT receptor antagonist) tended to exert the same effect as propranolol (Fig. 5D). Furthermore, baclofen ($GABA_B$ receptor agonist) and DNQX (non-NMDA-type glutamate receptor antagonist) did not suppress the TRH tartrate-induced increase in cGMP levels (Fig. 8C, Fig. 7B). Statistical analysis of the effect of baclofen was carried out using Student's t-test after a two-way ANOVA (baclofen factor in the superior vermis, $F(1,16) = 8.754$, $P < 0.01$, baclofen factor in the inferior vermis, $F(1,16) = 13.235$, $P < 0.01$, baclofen factor in the hemispheres, $F(1,16) = 9.544$, $P < 0.01$, TRH tartrate factor in the superior vermis, $F(1,16) = 36.626$, $P < 0.01$, TRH tartrate factor in the inferior vermis, $F(1,16) = 24.826$, $P < 0.01$, TRH tartrate factor in the hemispheres, $F(1,16) = 21.953$, $P < 0.01$, baclofen × TRH tartrate factor in the superior vermis, $F(1,16) = 0.805$, not significant, baclofen × TRH tartrate factor in the inferior vermis, $F(1,16) = 3.865$, not significant, baclofen × TRH tartrate factor in the hemispheres, $F(1,16) = 0.780$, not significant). In contrast, the TRH tartrate-induced increase in cGMP levels in various cerebellar areas was suppressed by pretreatment with muscimol ($GABA_A$ receptor agonist, Fig. 8A), THIP ($GABA_A$

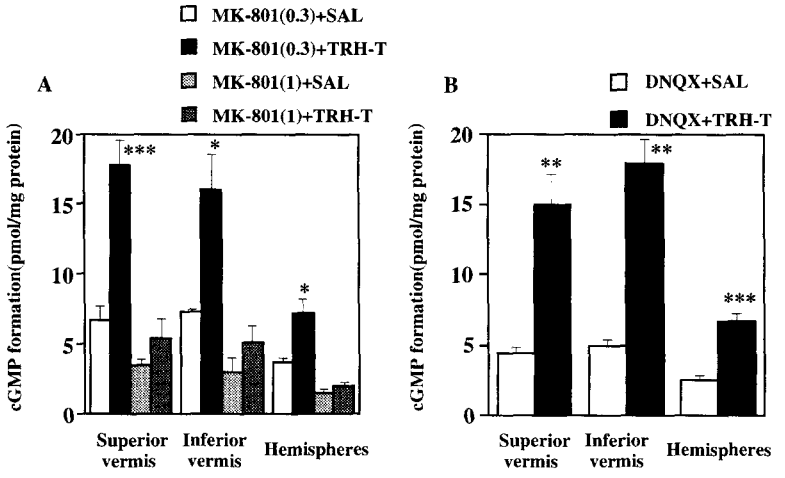

Fig. 7. Effects of glutamate receptor antagonists on TRH tartrate-induced cGMP formation in the cerebellar areas of the rat brain. (A) Dizocilpine (MK-801, 0.3 or 1 mg/kg, i.p.), (B) DNQX (30 mg/kg, i.p.) or saline (SAL) was given 30 min before injection of TRH tartrate (17 mg/kg, s.c. as free TRH). Rats were killed by focused microwave irradiation 10 min after injection of TRH tartrate or saline (SAL). cGMP levels in each cerebellar area were determined by a radioimmunoassay method. Each column represents the mean ± S.E. for 5 animals. * $P < 0.05$, *** $P < 0.001$ compared with MK-801 + SAL (Student's t-test following ANOVA), ** $P < 0.01$, *** $P < 0.001$ compared with DNQX + SAL (Student's t-test).

receptor agonist, Fig. 8B) or MK-801 (NMDA-type glutamate receptor antagonist, Fig. 7A) and partially suppressed by pretreatment with atropine (muscarinic receptor antagonist, Fig. 6). Statistical analysis of the effect of MK-801 was carried out using Student's t-test after a two-way ANOVA (MK-801 factor in the superior vermis, $F(1,15) = 37.931$, $P < 0.01$, MK-801 factor in the inferior vermis, $F(1,15) = 24.256$, $P < 0.01$, MK-801 factor in the hemispheres, $F(1,14) = 39.399$, $P < 0.01$, TRH tartrate factor in the superior vermis, $F(1,15) = 26.626$, $P < 0.01$, TRH tartrate factor in the inferior vermis, $F(1,15) = 12.545$, $P < 0.01$, TRH tartrate factor in the hemispheres, $F(1,14) = 13.758$, $P < 0.01$, MK-801 × TRH tartrate factor in the superior vermis, $F(1,15) = 12.035$, $P < 0.01$, MK-801 × TRH tartrate factor in the inferior vermis, $F(1,15) = 4.378$, $P < 0.05$, MK-801 × TRH tartrate factor in the hemispheres, $F(1,14) = 6.702$, $P < 0.05$).

4. Discussion

TRH tartrate induced a significant elevation of cGMP levels in the various cerebellar areas and brainstem. In particular, TRH tartrate had a marked effect on the cerebellar vermis as compared with cerebellar hemispheres. A projection from the vermis in the rat innervates the supe-

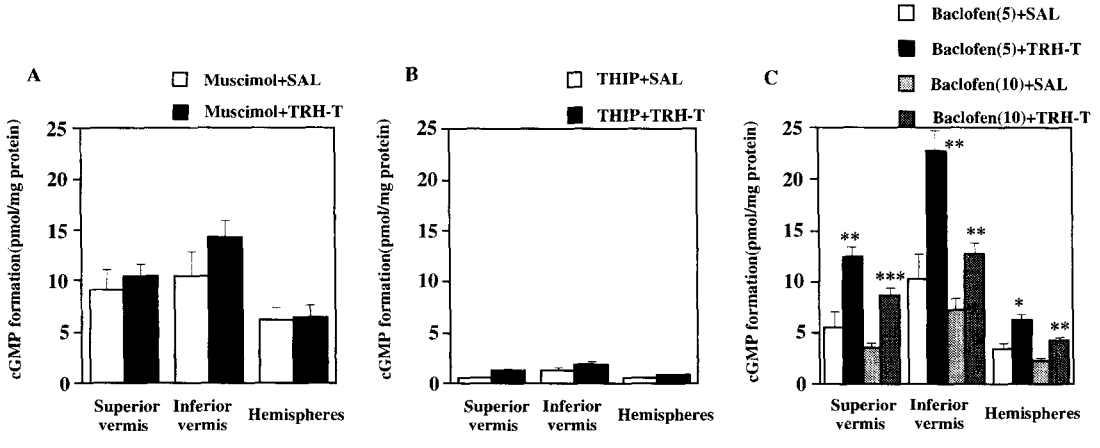

Fig. 8. Effects of GABA receptor agonists on TRH tartrate-induced cGMP formation in the cerebellar areas of the rat brain. (A) Muscimol (1 mg/kg, i.p.), (B) THIP (16 mg/kg, i.p.), (C) baclofen (5 or 10 mg/kg, i.p.) or saline (SAL) was given 30 min before injection of TRH tartrate (17 mg/kg, s.c. as free TRH). Rats were killed by focused microwave irradiation 10 min after injection of TRH tartrate or saline (SAL). cGMP levels in each cerebellar area were determined by a radioimmunoassay method. Each column represents the mean ± S.E. for 5 animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with baclofen + SAL (Student's t-test following ANOVA).

rior vestibular nucleus, which is caudally adjacent to the parabrachial nucleus in the brainstem. The vermis was reported to be involved in the control of posture, muscle tonicity and equilibrium functions (Chambers and Sprague, 1955). On the other hand, the cerebellar hemispheres receive a projection from the cerebral cortex via the pontine nucleus and were reported to modulate voluntary movement (Chambers and Sprague, 1955). Therefore, the amelioration of ataxic gait in various mutant animals by TRH tartrate may be due to the action mainly on the cerebellar vermis rather than the cerebellar hemispheres and other areas.

TRH tartrate-induced cGMP formation in the cerebellar areas was suppressed by treatment with MK-801, muscimol or THIP and partially suppressed by atropine but was not suppressed by chlordiazepoxide, oxazepam, phentolamine, propranolol, cyproheptadine, haloperidol, baclofen or DNQX.

Pazos et al. (1985) studied the TRH receptor site in rat brain by an autoradiographic method and found a significant concentration of specific binding in the medial part of the molecular layer in the simple lobule and lobule 5 of the cerebellum. They suggested that the TRH receptor in the cerebellum might be involved in the activation of phosphoinositides turnover. Moreover, Iriuchijima and Mori (1989) working with slices of rat brain reported that TRH caused a significant increase in the cAMP levels in the hypothalamus, striatum and midbrain and stimulated the formation of inositol phosphates in the cerebellum. Therefore, we used chlordiazepoxide, a TRH receptor antagonist (Sharif et al., 1983; Sharif and Burt, 1984; Simasko and Horita, 1982, Simasko and Horita, 1984) to investigate the possible involvement of the TRH receptor in the cGMP formation induced by TRH tartrate in the cerebellum. Cholordiazepoxide (30 mg/kg) did not suppress the TRH tartrate-induced increase in cGMP levels in the cerebellar areas. The TRH receptor in the cerebellum therefore does not seem to be involved in TRH tartrate-induced cGMP formation.

The levels of cGMP in the cerebellar cortex are thought to be regulated either by an excitatory input of climbing and mossy-parallel fibers or by an inhibitory input mediated by the activation of GABA receptors (Biggio et al., 1977a). The excitatory transmitter released from climbing and mossy fibers and from granule cell axons (parallel fiber) appears to be glutamate. Considerable evidence suggests that the second messenger of this transmitter is cGMP, most of which is produced in Purkinje cells (Biggio and Guidotti, 1976). The cGMP level would be increased when the excitatory input is facilitated or when stimulation of GABA receptors is decreased (Costa et al., 1975; Biggio et al., 1977b). Therefore, we investigated the possibility that TRH tartrate interacts directly with the glutamate system by stimulation of the climbing and/or mossy-parallel fibers. MK-801 and DNQX significantly decreased the basal levels of cGMP in the cerebellar areas (data not shown). The TRH tartrate-induced cGMP formation in various cerebellar areas was suppressed by pretreatment with MK-801 in a non-specific manner but not by DNQX, suggesting the possible involvement of NMDA-type glutamate receptors. TRH has been reported to enhance NMDA-elicited responses in the cerebral cortex, hippocampus and spinal cord (e.g., excitatory postsynaptic potential (EPSP), single motor unit reflex) (Kasparov et al., 1994; Stocca and Nistri, 1994, 1995; Chizh and Headley, 1994) and appears to increase the cGMP level in the cerebellum via the activation of NMDA receptors, which induces a Ca^{2+} influx through NMDA receptor-operated channels and subsequent stimulation of NO (nitric oxide) synthase (Garthwaite et al., 1988). NO stimulates soluble guanylyl cyclase and subsequently causes elevation of the cGMP level in the cerebellum (Garthwaite et al., 1988; Koesling et al., 1993; Schulz et al., 1991).

We have also found that $GABA_A$ receptor agonists, such as muscimol and THIP, but not a $GABA_B$ receptor agonist (baclofen) suppress TRH tartrate-induced cGMP formation, suggesting the possible involvement of $GABA_A$ receptors but not $GABA_B$ receptors. There have been several reports suggesting a positive relationship between cerebellar cGMP levels and motor function (Mueller et al., 1978; Lundberg et al., 1979), but we presented an exception to these reports, i.e., the finding that intraperitoneal treatment with muscimol ($GABA_A$ receptor agonist) at a dose of 1 mg/kg did not decrease the cerebellar cGMP level whereas it induced a severe change in motor function (data not shown), suggesting that all drugs that cause changes in motor function do not necessarily affect the cerebellar cGMP levels. Bowery (1989) reported that $GABA_B$ receptors predominate in the molecular layer, while $GABA_A$ receptors predominate in the granule cell layer in the cerebellum. The difference in the distribution of these GABA receptors in the cerebellum may contribute at least in part to the difference in responses to $GABA_A$ receptor agonists and $GABA_B$ receptor agonists. Our present results are consistent with the previous report by Cott et al. that the analeptic activity of TRH was antagonized by systemic administration of GABAergic drugs (Cott and Engel, 1977). These results suggest that TRH tartrate induces cGMP formation in the granule cell layer of the cerebellum.

Phentolamine significantly decreased the basal levels of cGMP in the cerebellar areas, but propranolol did not have significant effects (data not shown). These results coincide with the previous report showing that the increase in rat cerebellar cGMP levels caused by noradrenaline is mediated by α-adrenoceptors but not β-adrenoceptors (Haidamous et al., 1980). Phentolamine and propranolol did not suppress the effect of TRH tartrate. Haloperidol also caused a significant decrease in the basal levels of cGMP (data not shown) but did not suppress the effect of TRH tartrate. Cyproheptadine did not have a significant effect on the basal levels (data not shown) or on the TRH tartrate-in-

duced increase in cGMP levels. Therefore, monoaminergic neurotransmission may not be involved in the effect of TRH.

Additionally, we found that the muscarinic receptor antagonist, atropine, partially suppressed TRH tartrate-induced cGMP formation in the cerebellar areas. To date, cholinergic innervation in the cerebellum has not been conclusively demonstrated (Miyamoto et al., 1987). This may be due to the reciprocal interaction of cholinergic neurons with GABAergic neurons, i.e., compounds which have an inhibitory effect on GABA receptor agonists behave like cholinomimetics (Sarter and Bruno, 1994). For this reason, atropine may have suppressed the effect of TRH tartrate in the present study.

In conclusion, the present results suggest that excitatory amino acids, in particular NMDA, may be involved in the cGMP formation caused by TRH tartrate in the cerebellar areas and that cGMP formation is inhibited by activation of $GABA_A$ receptor function.

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