INCREASE IN RAT REGIONAL BRAIN CYCLIC NUCLEOTIDES BY THYROTROPIN-RELEASING HORMONE (TRH) AND ITS ANALOG DN-1417

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Abstract—The effects of thyrotropin-releasing hormone (TRH) and its analog γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate (DN-1417) on adenosine 3',5'-monophosphate (cyclic AMP) and guanosine 3',5'-monophosphate (cyclic GMP) levels in rat brain were investigated using a radioimmunoassay method. The time course of elevation of these nucleotides in various brain regions after administration of DN-1417 showed a peak at 5 to 15 min followed by a gradual decrease. DN-1417 (1 to 10 mg/kg i.p.) caused a dose-related increase in cyclic AMP levels in the cerebellum, cerebral cortex, striatum, nucleus accumbens, thalamus, hypothalamus and brain stem; whereas significant increases in cyclic GMP were observed in the cerebellum, nucleus accumbens and brain stem. TRH (3 to 10 mg/kg i.p.) caused significant increases of cyclic AMP in the cerebellum, cerebral cortex, nucleus accumbens, thalamus and brain stem and also caused an increase in cerebellar cyclic GMP. With one exception, DN-1417, apomorphine (Apo), methamphetamine (MAP) (all, 3 mg/kg i.p.)- and TRH (10 mg/kg i.p.)-induced increases in cyclic nucleotides were blocked by pimozide (1 mg/kg i.p., 4 hr before), a dopamine receptor blocker; the exception was a TRH-induced increase in cerebellar cyclic GMP. These increases were also blocked by propranolol (10 mg/kg i.p., 30 min before), an adrenergic β-receptor blocker, α-Methyl-p-tyrosine (α-MT, 250 mg/kg i.p., 4 hr before), a tyrosine hydroxylase inhibitor, almost completely blocked DN-1417- and MAP-induced increases in cyclic nucleotides, slightly blocked TRH-effects, and had no effect on Apo-effects. These in vivo results were confirmed in an in vitro system using brain slices. The addition of DN-1417 (10^{-4} M) or TRH (10^{-3} M) significantly enhanced the spontaneous[^3H]-dopamine and[^3H]-norepinephrine release from the superfused slices of the rat nucleus accumbens and cerebral cortex in vitro. The addition of DN-1417 (10^{-4} M) or TRH (10^{-4} M) had no effect on the activities of adenylate cyclase and guanylate cyclase, although only a high concentration (10^{-3} M) of DN-1417 inhibited the cyclic AMP- and cyclic GMP-hydrolytic activities in various brain region homogenates. These results suggest that DN-1417 does not produce an increase in the levels of cyclic nucleotides by direct receptor-enzyme activation, but that DN-1417 like MAP causes the increase through endogenous catecholaminergic, particularly dopaminergic activation.

Among a number of newly synthesized TRH analogs, our co-workers found that γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate (DN-1417) has a more pronounced effect on the central nervous system (CNS) than does TRH in antagonizing pentobarbital-induced sleep in mice, yet it has a thyrotropin (TSH)-releasing activity of approximately one-fortieth that of TRH in rats (1). More detailed neuropharmacological
actions of DN-1417 compared with those of TRH have been presented (2).

Cyclic nucleotides are putative intracellular messengers of the actions of neurotransmitters within the CNS. DN-1417-induced spontaneous motor hyperactivity and antipentobarbital action have been postulated to be relevant to the monoaminergic, especially the dopaminergic system, and the cholinergic system, respectively (3, 4). Several studies have indicated that monoaminergic agonists increase the content of adenosine 3',5'-monophosphate (cyclic AMP) and cholinergic agonists increase guanosine 3',5'-monophosphate (cyclic GMP) levels. The present studies were undertaken to examine the mechanism by which TRH and DN-1417 alter the brain cyclic nucleotide system in regions in which monoaminergic and cholinergic nerve terminals are present and to observe the effects of dopaminergic receptor and adrenergic β-receptor blockers on TRH- and DN-1417-induced increases in cyclic nucleotide levels.

Materials and Methods

Animals: Male Sprague-Dawley (SD) rats were purchased from Japan Clea Laboratories, and they were used when they weighed 250 to 300 g (8 weeks old) after a minimum of 3 days of acclimation to our animal facilities (12-hr light, 12-hr dark cycle).

Cyclic nucleotide assay in vivo: Rats were exposed to microwaves in a Toshiba Microwave Applicator (TMW 6402 A) focused microwave oven delivering 5 KW power for 1.2 or 1.5 sec, depending on the animal's size. The head was removed, cooled in ice-water bath for 30 min, and the brain was dissected into 7 regions: cerebral cortex, striatum, nucleus accumbens, thalamus, hypothalamus, brain stem and cerebellum, using the procedure of Glowinski and Iversen (5). The tissues were then homogenized in 1 ml of 5% trichloroacetic acid (TCA) using a polytron, and the homogenates were centrifuged at 2,000 g for 10 min. The supernatants were mixed with water saturated ether three times to eliminate the TCA. The ether evaporated supernatants were assayed directly for cyclic AMP and cyclic GMP by the radioimmunoassay method using Yamasa Assay Kits. The pellets were dissolved in 4% sodium hydroxide for protein measurements using the Folin reagent (6).

Cyclic nucleotide assay in vitro: Cyclic AMP and cyclic GMP formations in the slices were determined according to the method of Krueger et al. (7). The brain regions were dissected and suspended in ice-cold Krebs-Ringer phosphate buffer (KRPB) containing 10 mM theophylline. The 2.5 mm thick slices were chopped with a Mcllwain tissue chopper, suspended in KRPB, centrifuged at 1,000 g for 10 min, and rinsed with KRPB. The wet slices were weighed and resuspended in fresh KRPB to a final volume of 50 mg/ml. A 0.5 ml aliquot of these suspensions were added to tubes containing 0.1 ml KRPB with various drugs, and the preparations were incubated for 15 min at 37°C. After the reaction was terminated by placing the tube in boiling water for 3 min, the supernatant obtained by centrifugation (1,000 g for 10 min) was assayed for cyclic AMP and cyclic GMP by the radioimmunoassay method using Yamasa Assay Kits.

Adenylate cyclase and guanylate cyclase assay: Adenylate cyclase and guanylate cyclase activities of various brain regions were measured in decapitated rats. The tissue samples for the enzyme assay were homogenized at 4°C in 10 volumes of 50 mM Tris-chloride (pH 7.5) with a Potter glass-Teflon homogenizer. A 25 μl aliquot of this homogenate was incubated at 37°C for 3 min in 0.2 ml of 80 mM Tris-maleate buffer (pH 7.4) containing 0.5 mM ATP or GTP, 2 mM MgSO₄ and 10 mM theophylline with or
without 25 μl drug solution. The reaction was stopped by boiling for 3 min, and 12.5 μl of the supernatant (2,000 g for 10 min) of the reaction mixture was assayed for cyclic AMP and cyclic GMP levels by the radio-immunoassay technique using Yamasa Assay Kits. To determine endogenous cyclic AMP and cyclic GMP, the enzyme assay was also carried out without the addition of 0.5 mM ATP or GTP.

Phosphodiesterase assay: Various sections of the brain were homogenized in 10 volumes of 50 mM Tris-chloride (pH 7.5) at 4°C with a Potter glass-Teflon homogenizer. The cyclic AMP- and cyclic GMP-hydrolytic activities were assayed according to the method of Thompson and Appleman (8). One hundred μl of this homogenate was incubated at 37°C for 20 min in 350 μl of 50 mM Tris-chloride buffer (pH 7.5) containing 1 mM [3H]-cyclic AMP or [3H]-cyclic GMP (30,000 dpm/0.1 μmol), with or without 50 μl of drug solution. The reaction was stopped by boiling for 5 min, kept cool in an ice-water bath, incubated for a further 10 min with 0.1 ml of snake venom (1 mg/ml), and stopped by 1 ml of Bio-Rad AG 1 × 2 suspension (resin 1: water 2). A 0.5 ml aliquot of the supernatant (780 g for 15 min) of the reaction mixtures was counted by ACS® II liquid scintillation.

Release of [3H]-dopamine (DA) and [3H]-norepinephrine (NE) from the slices: After decapsulation, the rat nucleus accumbens and cerebral cortex were quickly dissected out and then sliced to a thickness of 0.5 mm with a McIlwain tissue chopper. The slices were dispersed in 95% O2-5% CO2 saturated KRPB containing 0.1 mM pargyline, 1 mM ascorbic acid and 10 mM glucose, and were centrifuged at 2,000 g for 6 min at 4°C. The slices of 40 mg wet weight were put into each test tube containing 2 ml KRPB, incubated with 1 μCi/3.7 × 10⁻⁸ M of [3H]-DA or 1 μCi/3.3 × 10⁻⁹ M of L-[7,8-³H(N)]-NE ([³H]-NE) for 60 min at 37°C, then repeatedly rinsed with fresh KRPB and centrifuged three times.

The slices preloaded with [³H]-DA or [³H]-NE were superfused with KRPB at a constant rate of 0.2–0.3 ml/min in a superfusion apparatus. The radioactivities in the superfusate effluents collected every 10 min for 30 min and every 5 min from 30 to 100 min, and those in the supernatant (3,000 g for 10 min at 4°C) of 5% TCA homogenized slices were counted using a liquid scintillation spectrometer with ACS® II scintillation.

Agents used: r-Butyrolactone-r-carbonyl-L-histidyl-L-prolinamide citrate (DN-1417, Lot No. I 615-02), 2-hydroxy-4-carboxybutanoyl-L-histidyl-L-prolinamide (Lot No. I 615-ML-2FD), thyrotropin-releasing hormone tartrate (TRH, Lot No. T-8-2), methamphetamine HCl (MAP, Dainippon), apomorphine HCl (August Brandes), DL-propranolol HCl, dopamine HCl, L-norepinephrine HCl, snake venom (Sigma), DL-α-methyl-p-tyrosine (α-MT, Aldrich), ATP and GTP (Boehringer Mannheim), trichloroacetic acid (TCA), Tris, maleic acid, MgSO4, citric acid, tartaric acid, acetylcholine chloride (Wako), theophylline HCl (Tokyo Kasei), ACS® II (Amersham), cyclic AMP and cyclic GMP Assay Kits (Yamasa Shoyu), [³H]-cyclic AMP, [³H]-cyclic GMP, [³H]-dopamine and [³H]-norepinephrine (New England Nuclear).

Statistics: Statistical comparisons between different treatments were made using Student's t-test (two-tailed).

Results
Effects on cyclic AMP and cyclic GMP levels in various regions of the rat brain in vivo
A) Time course: Changes with time from 5 to 60 min in cyclic AMP and cyclic GMP levels of the cerebellum, cerebral cortex, striatum, nucleus accumbens, thalamus, hypothalamus and brain stem were examined in
rats receiving DN-1417 (3 mg/kg), TRH (3 mg/kg) or methamphetamine (MAP, 1 mg/kg) intraperitoneally (i.p.). Figure 1 shows typical data obtained from thalamic tissues. The time course of these changes indicated that i.p. administration of DN-1417, TRH or MAP caused peaks between 5 to 15 min followed by decreases to values not

Fig. 1. Changes with time in cyclic AMP and cyclic GMP levels in the rat thalamus. Saline (■), DN-1417 (○, 3 mg/kg), TRH (●, 3 mg/kg), or methamphetamine (MAP) (△, 1 mg/kg) was administered intraperitoneally (i.p.) 0, 5, 15, 30 and 60 min before microwave irradiation (5 KW, 1.5 sec). Cyclic AMP (—) and cyclic GMP (---) were determined by the radioimmunoassay method using Yamasa Assay Kits. All points are the mean±S.E. of 4 experiments. *P<0.05, ***P<0.001 vs. saline group.

Fig. 2. Effects of DN-1417 and TRH on cyclic AMP (cAMP) and cyclic GMP (cGMP) levels in various regions of the rat brain. DN-1417 (○) or TRH (●, both 1, 3, 10 mg/kg) was administered i.p. 5 min before microwave irradiation (5 KW, 1.5 sec). Each value is shown as a percent increase of the saline group (100%) and is the mean±S.E. of 8 experiments. *P<0.05, **P<0.01, ***P<0.001 vs. saline group.
significantly different from the control group 30 or 60 min after the injection. Thus the following dose-response experiments were done at 5 and 15 min after administration.

B) Dose-response: Five min after 1 mg/kg of DN-1417 was administered, cyclic AMP levels increased in the cerebellum, thalamus and brain stem. Three mg/kg further increased cyclic AMP levels in the cerebral cortex, striatum, nucleus accumbens and hypothalamus, and cyclic GMP levels in the cerebellum and brain stem; 10 mg/kg gave the same results as 3 mg/kg (Fig. 2).

Fifteen min after 3 mg/kg of DN-1417 was administered, cyclic AMP levels increased in the cerebellum. Ten mg/kg increased cyclic AMP levels in the cerebral cortex, cerebellum and nucleus accumbens, and it increased cyclic GMP levels in the cerebellum, cerebral cortex and striatum; 30 mg/kg increased cyclic AMP levels in the striatum, nucleus accumbens and brain stem, and it increased cyclic GMP levels in the cerebellum, cerebral cortex and brain stem. 2-Hydroxy-4-carboxybutanoyl-L-histidyl-L-prolinamide, a main metabolite of DN-1417, at 20 mg/kg and citric acid, a salt constituent of DN-1417, at 12 mg/kg which was equimolar to 30 mg/kg of DN-1417 had no effect on the levels of these nucleotides.

Five min after 3 mg/kg of TRH was administered, cyclic AMP increased in the cerebellum and thalamus as did cerebellar cyclic GMP; 10 mg/kg increased cyclic AMP in the cerebellum, cerebral cortex, nucleus accumbens and brain stem, and it increased cerebellar cyclic GMP (Fig. 2).

Fifteen min after 10 mg/kg of TRH was administered, cyclic AMP increased in the cerebral cortex and nucleus accumbens as did cerebellar cyclic GMP. Thirty mg/kg increased cyclic AMP in the cerebral cortex and brain stem, and it increased cyclic GMP in the cerebellum, cerebral cortex and brain stem. Tartaric acid, a salt constituent of TRH, at 9 mg/kg which was equimolar to

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Fig. 3. Effects of pimozide (a dopamine receptor blocker) or α-methyl-p-tyrosine (α-MT, a tyrosine hydroxylase inhibitor) on DN-1417, TRH, methamphetamine (MAP) and apomorphine (Apo)-induced elevations of cyclic AMP (cAMP) and cyclic GMP (cGMP) levels in the cerebellum and cerebral cortex. Saline, DN-1417 (3 mg/kg), TRH (10 mg/kg), MAP (3 mg/kg) or Apo (3 mg/kg) was administered i.p. 5 min before killing. Pimozide (1 mg/kg suspended in 3% gum arab.) or α-MT (250 mg/kg) was administered i.p. 4 hr before the drug treatments. All values represent the mean±S.E. of 6 experiments. *P<0.05, **P<0.01, ***P<0.001 vs. saline group.
30 mg/kg of TRH had little effect on cyclic AMP and cyclic GMP levels.

Effects of various blockers on TRH- or DN-1417-induced increases of cyclic AMP and cyclic GMP in rat brain in vivo

A) Dopamine receptor blocker: As shown in Figs. 3 and 4, five min after 3 mg/kg of DN-1417, MAP and a dopaminergic receptor agonist, apomorphine, were administered, cyclic AMP increased in all 7 brain regions; and TRH (10 mg/kg i.p.) increased cyclic AMP in the cerebellum, cerebral cortex, nucleus accumbens and brain stem. The same treatment with DN-1417, MAP, apomorphine and TRH increased cerebellar cyclic GMP.

Pimozide, a dopamine receptor blocker, (1 mg/kg i.p.) administered 4 hr before the DN-1417, TRH, apomorphine or MAP dosing, almost completely abolished the drug-induced increases in cyclic AMP levels, except for the TRH-effect in cerebellar cyclic GMP.

B) Adrenergic β-receptor blocker: Propranolol, an adrenergic β-receptor blocker, (10 mg/kg i.p.) administered 30 min before DN-1417, TRH or apomorphine had little effect on the increases in cyclic AMP and cyclic GMP induced by these drugs.

C) Tyrosine hydroxylase inhibitor: As shown in Figs. 3 and 4, α-methyl-p-tyrosine (α-MT), a tyrosine hydroxylase inhibitor, (250 mg/kg i.p.) administered 4 hr before the drugs almost completely abolished DN-1417- and MAP-induced increases in cyclic AMP and cyclic GMP levels, but had no effect on the apomorphine-effect.

Effects on the release of [3H]-dopamine (DA) and [3H]-norepinephrine (NE) from the slices of the nucleus accumbens and cerebral cortex

Effects of DN-1417 and TRH on the release of [3H]-DA and [3H]-NE from the slices of the nucleus accumbens and cerebral cortex were investigated by means of the superfusion method. As shown in Fig. 5(a), DN-1417 (10⁻⁵–10⁻⁴ M) and TRH (10⁻⁴–10⁻³ M) concentration-dependently enhanced the release of [3H]-DA from the superfused nucleus accumbens slices after a stable spontaneous DA release was established. Additions of DN-1417 (10⁻⁵–
10^{-3} M) and TRH (10^{-4}-10^{-2} M) also enhanced the release of [3H]-NE from the superfused cerebral cortex slices. KCl (5 \times 10^{-2} M) stimulated both releases to the same extent.

These results suggest that both DN-1417 and MAP increase the levels of cyclic nucleotides in the brain through catecholaminergic, particularly dopaminergic activation.

**Effects on cyclic AMP and cyclic GMP formation in the slices of the nucleus accumbens and cerebellum in vitro**

Additions of DN-1417 (10^{-6}-10^{-4} M), TRH (10^{-5}-10^{-3} M), NE (10^{-4} M) or DA (10^{-4} M) stimulated cyclic AMP formation in the slices of the cerebral cortex, limbic area (hypothalamus, thalamus and nucleus accumbens) and cerebellum in a concentration-dependent manner. As shown in Table 1, propranolol (10^{-4} M) almost completely abolished NE- and DA-induced cyclic AMP formation, but did not block TRH- and DN-1417-induced effects. Pimozide (10^{-4} M) almost completely abolished TRH- and DN-1417-induced cyclic AMP formation, but not the NE-induced effect (Table 1).

Additions of DN-1417 (10^{-6}-10^{-4} M), TRH (10^{-6}-10^{-3} M) and carbachol (10^{-4} M) stimulated cyclic GMP formation in the
Table 1. Effects of propranolol and pimozide on DN-1417-, TRH-, dopamine (DA)- and norepinephrine (NE)-induced cyclic AMP formation in rat nucleus accumbens slices

| Treatment (M) | Cyclic AMP formation (pmol/mg protein/15 min) |
|--------------|---------------------------------------------|
|              | None                                       |
| Control      | 18.8±1.0                                   |
| DN-1417      | 32.7±2.3***                                |
| TRH          | 29.0±2.1***                                |
| NE           | 36.6±6.9***                                |
| DA           | 32.3±3.8**                                 |
| Propranolol (10⁻⁴ M) | 23.0±1.3   |
| DN-1417      | 38.0±5.8*                                 |
| TRH          | 37.2±6.1*                                 |
| NE           | 21.6±3.2                                  |
| DA           | 27.4±3.1                                  |
| Pimozide (10⁻⁴ M) | 17.9±2.2   |
| DN-1417      | 28.7±5.3                                  |
| TRH          | 22.3±3.3                                  |
| NE           | 34.4±4.2*                                 |
| DA           | 23.6±3.3                                  |

*P<0.05, **P<0.01, ***P<0.001 when compared to each control group. Each value is the mean±S.E. of 6 experiments.

Table 2. Effects of propranolol and pimozide on DN-1417-, TRH-, dopamine (DA)-, norepinephrine (NE)- and carbachol (Carb)-induced cyclic GMP formation in rat cerebellar slices

| Treatment (M) | Cyclic GMP formation (pmol/mg protein/15 min) |
|--------------|---------------------------------------------|
|              | None                                       |
| Control      | 13.1±1.3                                   |
| DN-1417      | 20.9±1.4**                                 |
| TRH          | 30.0±8.7*                                  |
| NE           | 12.8±1.6                                   |
| DA           | 15.8±1.8                                   |
| Carb         | 21.2±1.5**                                 |
| Propranolol (10⁻⁴ M) | 11.5±0.9   |
| DN-1417      | 15.0±2.1*                                 |
| TRH          | 15.0±1.0*                                 |
| NE           | 11.6±0.6                                  |
| DA           | 14.4±1.5                                  |
| Carb         | 17.6±1.2**                                 |
| Pimozide (10⁻⁴ M) | 12.8±1.1   |
| DN-1417      | 14.7±1.3                                  |
| TRH          | 15.0±1.3                                  |
| NE           | 15.0±1.0                                  |
| DA           | 12.2±0.9                                  |
| Carb         | 14.0±1.8                                  |

*P<0.05, **P<0.01 when compared to each control group. Each value is the mean±S.E. of 6 experiments.

slices of the cerebellum. As shown in Table 2, propranolol (10⁻⁴ M) had no effect on the DN-1417-, TRH- and carbachol-effects. Pimozide almost completely abolished the TRH-, DN-1417- and carbachol-effects.

These in vitro results confirm the above-mentioned in vivo results.

Effects on the activities of adenylate cyclase, guanylate cyclase and phosphodiesterase in the rat brain homogenates

A) Adenylate cyclase (AC) and guanylate cyclase (GC): As shown in Table 3, additions of DN-1417 (10⁻⁴ M) and TRH (10⁻⁴ M) had no effect on the activities of AC in the rat brain homogenates, while the addition of DA (10⁻⁴ M) stimulated the AC activities of the striatum and nucleus accumbens homogenates; and the addition of NE (10⁻⁴ M) stimulated the activities in the cerebral cortex, thalamus and hypothalamus homogenates.

DN-1417 (10⁻⁴ M) and TRH (10⁻⁴ M) had no effect on the GC activity of the cerebellar homogenates, while the addition of acetylcholine (10⁻⁴ M) stimulated GC activity (Table 4).

B) Phosphodiesterase: With concentrations from 10⁻⁵ to 10⁻³ M of DN-1417 and theophylline, there was a concentration-dependent inhibition of the cyclic AMP-hydrolytic activities in the nucleus accumbens and hypothalamus homogenates, while the addition of acetylcholine (10⁻⁴ M) stimulated GC activity (Table 5). The potency of DN-1417 was about one-tenth that of theophylline.

The same concentration of DN-1417 and theophylline also markedly inhibited the cyclic GMP-hydrolytic activity in the cerebellar homogenates, while TRH had no...
Table 3. Effects of DN-1417, TRH, norepinephrine (NE) and dopamine (DA) on the activity of adenylate cyclase in various brain homogenates of rats

| Treatment | Concentration (M) | Cerebral cortex | Striatum | Nuc. accumbens | Thalamus | Hypothalamus | Brain stem |
|-----------|------------------|----------------|----------|----------------|----------|-------------|-----------|
| Control   |                  | 73.5±2.9 (100%) | 54.8±3.5 (100%) | 48.0±3.7 (100%) | 55.3±4.9 (100%) | 38.5±3.4 (100%) | 23.7±2.8 (100%) |
| DN-1417   | 10⁻⁴             | 75.5±5.9 (103) | 67.7±5.8 (123) | 48.5±5.9 (101) | 66.1±4.4 (120) | 54.1±10.5 (141) | 20.5±1.5 (86) |
| TRH       | 10⁻⁴             | 77.8±7.5 (108) | 67.7±5.8 (97) | 49.8±3.7 (104) | 64.4±6.8 (117) | 44.7±6.2 (116) | 23.5±2.4 (99) |
| DA        | 10⁻⁴             |                | 77.0±8.0* (140) | 67.7±9.2* (141) |          |             |           |
| NE        | 10⁻⁴             | 128.8±19.4* (170) |          |             | 76.2±8.3* (138) | 76.0±5.7*** (197) | 32.9±4.4 (139) |

*The homogenates were incubated with 0.5 mM ATP, 2 mM MgSO₄ and 10 mM theophylline in 80 mM Tris-maleate buffer (pH 7.4) at 37°C for 3 min. *P<0.05, **P<0.001 when compared to each control group. Each value is the mean±S.E. of 6 experiments.

Table 4. Effects of DN-1417, TRH and acetylcholine (ACh) on the activity of guanylate cyclase in rat cerebellar homogenates

| Treatment | Concentration (M) | Guanylate cyclase activity¹ (pmol/mg protein/min) | % |
|-----------|------------------|-----------------------------------------------|---|
| Control   |                  | 4.0±0.7 (100)                                |   |
| DN-1417   | 10⁻⁶             | 4.5±0.8 (113)                                |   |
|           | 10⁻⁵             | 3.8±0.7 (95)                                 |   |
|           | 10⁻⁴             | 4.0±0.9 (109)                                |   |
| TRH       | 10⁻⁶             | 4.9±0.9 (123)                                |   |
|           | 10⁻⁵             | 3.8±0.8 (95)                                 |   |
|           | 10⁻⁴             | 4.5±113 (113)                                |   |
| ACh       | 10⁻⁵             | 4.1±0.6 (103)                                |   |
|           | 10⁻⁴             | 7.4±1.3* (185)                               |   |

¹The homogenates were incubated with 0.5 mM GTP, 2 mM MgSO₄ and 10 mM theophylline in Tris-maleate buffer (pH 7.4) at 37°C for 3 min. *P<0.05 when compared to the control group. Each value is the mean±S.E. of 6 experiments.

Therefore, while DN-1417 does not enhance the activities of AC and GC directly, its highest concentration (10⁻³ M) inhibits cyclic AMP- and cyclic GMP-hydrolytic activities.

Discussion

A TRH analog, DN-1417 (1 to 10 mg/kg i.p.), caused a dose related-increase in cyclic AMP levels in the brain stem, thalamus, cerebellum, nucleus accumbens, hypothalamus, cerebral cortex and striatum; cyclic GMP levels in the cerebellum and brain stem also increased. However, citric acid, a salt constituent of DN-1417, and 2-hydroxy-4-carboxybutanoyl-L-histidyl-L-prolinamide, the main metabolite of DN-1417, had little or no effect on the cyclic nucleotide levels. Therefore, DN-1417-induced cyclic AMP
Table 5. Effects of DN-1417, TRH, citric acid and theophylline on the cyclic AMP-hydrolytic activities in nucleus accumbens and hypothalamus homogenates of rats

| Treatment   | Concentration (M) | Cyclic AMP-hydrolytic activity<sup>13</sup> (nmol/mg protein/min) |
|-------------|------------------|---------------------------------------------------------------|
|             |                  | Nuc. accumbens % | Hypothalamus % |
| Control     |                  |                 |                |
| DN-1417     | 10<sup>-5</sup>  | 18.9±0.6 (100)  | 12.2±1.0 (100) |
|             | 10<sup>-4</sup>  | 20.5±0.8 (108)  | 14.1±0.7 (116) |
|             | 10<sup>-3</sup>  | 17.7±1.4 (94)   | 10.1±0.5 (83)  |
|             | 10<sup>-6</sup>  | 12.9±1.4*** (68) | 8.3±0.6** (68) |
| TRH         | 10<sup>-5</sup>  | 16.1±1.0 (95)   | 14.2±0.6 (116) |
|             | 10<sup>-4</sup>  | 16.8±0.7 (89)   | 13.6±0.5 (111) |
|             | 10<sup>-3</sup>  | 17.4±0.7 (92)   | 12.4±0.5 (102) |
| Theophylline | 10<sup>-6</sup>  | 18.0±1.0 (95)   | 11.9±0.7 (98)  |
|             | 10<sup>-4</sup>  | 14.6±0.5*** (77) | 9.4±0.3* (77) |
|             | 10<sup>-3</sup>  | 8.0±0.4*** (32)  | 6.0±0.5*** (49) |
| Citric acid   | 10<sup>-3</sup>  | 20.0±0.9 (106)   | 15.4±0.8 (126) |

<sup>13</sup>The homogenates were incubated with [3H]-cyclic AMP (25,000 dpm/0.1 μmol) at 37°C for 20 min. *P<0.05, **P<0.01, ***P<0.001 when compared to each control group. Each value is the mean±S.E. of 16 experiments.

Table 6. Effects of DN-1417, TRH and theophylline on the cyclic GMP-hydrolytic activity in rat cerebellar homogenates

| Treatment   | Concentration (M) | Cyclic GMP-hydrolytic activity<sup>13</sup> (nmol/mg protein/min) |
|-------------|------------------|---------------------------------------------------------------|
|             |                  |                  | %                |
| Control     |                  | 12.8±0.9 (100)  |                 |
| DN-1417     | 10<sup>-5</sup>  | 10.3±0.7 (80)   |                 |
|             | 10<sup>-4</sup>  | 5.3±0.5*** (41) |                 |
|             | 10<sup>-3</sup>  | 4.9±0.7*** (38) |                 |
| TRH         | 10<sup>-5</sup>  | 10.9±1.2 (85)   |                 |
|             | 10<sup>-4</sup>  | 10.6±0.9 (83)   |                 |
|             | 10<sup>-3</sup>  | 10.4±0.8 (81)   |                 |
| Theophylline | 10<sup>-3</sup>  | 3.0±0.2*** (23)  |                 |

<sup>13</sup>The homogenates were incubated with [3H]-cyclic GMP (31,000 dpm/0.1 μmol) at 37°C for 20 min. ***P<0.001 when compared to the control group. Each value is the mean±S.E. of 8 experiments.

and cyclic GMP increases seem to depend on the intact peptide structure of DN-1417 itself. Under the same conditions, the administration of TRH (1 to 10 mg/kg) increased cyclic AMP levels in the cerebellum, cerebral cortex, nucleus accumbens and brain stem, and it increased cyclic GMP levels in the cerebellum and brain stem. The DN-1417 effects were similar to those seen with TRH, but the potency of DN-1417 was about 3 to 10 times that of TRH. The catecholamine releaser MAP and the dopaminergic receptor agonist apomorphine (both, 1 to 3 mg/kg i.p.) increased cyclic AMP levels in all 7 brain regions and also increased cerebellar cyclic GMP levels. These results were consistent with the findings in the case of DN-1417. Pretreatment with α-MT, a tyrosine hydroxylase inhibitor, and pimozide, a dopamine receptor blocker, almost completely blocked the DN-1417-effect; propranolol, an adrenergic β-receptor blocker,
had little effect on the DN-1417-effect. The effects of these blockers on DN-1417 were similar to those on MAP. This result is consistent with the present in vitro results and support our other observation that DN-1417 stimulated the release of preloaded $[^3H]$-DA or $[^3H]$-NE from the nucleus accumbens or cerebral cortex in a manner same as that of MAP. In fact, the brain regions assayed in the present experiment contained dopaminergic nerve terminals, i.e., the nucleus accumbens and striatum, and noradrenergic terminals, i.e., the cerebral cortex and hypothalamus. Thus, DN-1417 like MAP seems to produce increases in brain cyclic nucleotides through catecholaminergic, particularly dopaminergic activation in vivo and in vitro.

Mailman et al. reported that TRH (30 mg/kg i.p.) caused a significant increase in the rat cerebellar and brain stem cyclic GMP 15 min after administration, whereas there was no significant increase in the cyclic GMP or cyclic AMP of other brain regions (9). We found that TRH significantly increased cyclic GMP levels in the cerebellum, brain stem and cerebral cortex, and it increased cyclic AMP levels in the cerebral cortex, hypothalamus and brain stem. Hence, our observations differ from those of Mailman et al. These same authors also reported that TRH caused a similar increase in the cerebellar cyclic GMP content in hypophysectomized or haloperidol pretreated rats, indicating that the increase is independent of the integrity of pituitary and dopaminergic transmission. These findings support our observations that DN-1417, which had about one-fortieth the TSH-releasing activity of TRH in rats, had a more potent cyclic nucleotide increasing activity than did TRH, and that pimozide did not block TRH-induced cyclic GMP increases in the cerebellum in vivo.

Biggio et al. reported that apomorphine increased cerebellar cyclic GMP levels and that pretreatment with haloperidol or intra-striatal injection of kainic acid blocked apomorphine-induced cerebellar cyclic GMP increases (10). These results indicate that dopaminergic mechanisms in the striatum are involved in the regulation of cerebellar cyclic GMP. Meyerhoff et al. suggested that locomotor activity contributed to elevations in cerebellar cyclic GMP and that forced immobilization decreased cerebellar cyclic GMP and attenuated the apomorphine-induced elevation (11). Breese et al. reported that apomorphine, amphetamine, and methylphenidate increased the cerebellar cyclic GMP; and other drugs proposed to be dopaminergic agonists such as bromocriptine, letofuril and piribedil, which induced locomotor activity, did not alter the levels of cyclic GMP in the cerebellum (12). It is, therefore, unclear whether or not secondary effects such as alterations in behavioral activity can contribute to the magnitude of change observed in the cerebellar cyclic GMP content after drug administrations. However, we had in vitro results that TRH and DN-1417 stimulated cyclic GMP formation in the cerebellar slices, indicating that TRH and DN-1417 had a direct effect on cyclic GMP levels in the cerebellum. TRH and DN-1417 had no effect on the GC activity of the cerebellar homogenates. These results indicate that the effects of TRH and DN-1417 need an intact cell membrane to act; the slices had intact membranes and the homogenates did not.

We found that pretreatment with pimozide blocked the DN-1417-induced cerebellar cyclic GMP elevation but did not block the TRH-induced effect, indicating that the actions of these drugs involved different mechanisms with regard to the cerebellar cyclic GMP elevation in vivo.

DN-1417 increases cyclic AMP in the cerebellum, cerebral cortex, striatum, nucleus accumbens, thalamus, hypothalamus and
brain stem, and it also increases cyclic GMP in the cerebellum and nucleus accumbens; these changes do not appear to be related to either activation of adrenergic β-receptors or to direct activation of adenylate cyclase or guanylate cyclase by DN-1417. DN-1417 seems to exert some central nervous system actions by increasing the levels of these nucleotides through endogenous dopaminergic activation. However, these activation mechanisms are very complicated, and further study is needed to solve this problem.

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