Effect of Alkylpyrazine Derivatives on the Duration of Pentobarbital-Induced Sleep, Picrotoxin-Induced Convulsion and γ -Aminobutyric Acid (GABA) Levels in the Mouse Brain

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The effect of alkylpyrazine derivatives on pentobarbital-induced sleeping time, picrotoxin-induced convulsion and γ -aminobutyric acid (GABA) levels in mouse brain were studied. The duration of pentobarbital-induced sleep in mice was dose-dependently increased by 2,5-dimethylpyrazine (DMP). The duration of pentobarbital-induced sleep was also increased by an administration route of intracerebroventricular injection. Sleep duration was also increased by the administration of isomers of DMP, 2-chloro-3,6-dimethylpyrazine (DMP-Cl) and 2-fluoro-3,6-dimethylpyrazine (DMP-F), but 3,6-dimethylpyrazine-2-thiol (DMP-SH) did not affect sleep duration. The interval until the appearance of picrotoxin-induced convulsion was prolonged by DMP and DMP-Cl. Increased sleep duration was obtained by administering DMP in combination with aminooxyacetic acid (AOAA) and diazepam compared to a single injection. The interval until convulsion due to picrotoxin was also prolonged by the administration of DMP combined with diazepam and valproic acid (VPA). The interval until the appearance of bicuculline-induced convulsion was also prolonged by pretreatment with DMP. The GABA level in mouse brain was increased by the administration of AOAA, VPA, DMP and DMP-Cl. These results suggest that DMP and other derivatives may strengthen the GABAergic system in the brain.

Key words 2,5-dimethylpyrazine; pentobarbital-induced sleep; picrotoxin-induced convulsion; γ -aminobutyric acid; pyrazine derivative

Alkylpyrazine derivatives have been observed as components of coffee, tea, cocoa, natto and soy bean sauce and other substances used for flavor,^{1–3)} and have been identified as biologically active components. 2,5-Dimethylpyrazine (DMP) and tetramethylpyrazine (TMP) were also observed, respectively in the adrenal gland⁴⁾ and in the Chinese traditional medicine “senkyuu”.⁵⁾ In China, TMP has been used as a medication to treat cerebrothrombosis or to increase coronary blood flow. We previously reported that DMP or DMP isomers caused a decrease in reproductive or accessory reproductive organ function in male or female rats.^{6–9)} In another investigation, one of the pyrazine derivatives, 5-chloro-2,3-bis(*p*-methoxy phenyl) pyrazine, more strongly inhibited platelet aggregation than aspirin.¹⁰⁾ Recently, we discovered another effect of DMP and its derivatives. DMP, 2-chloro-3,6-dimethylpyrazine (DMP-Cl) and 2-fluoro-3,6-dimethylpyrazine (DMP-F) extended the interval before the appearance of picrotoxin-induced convulsion and prolonged the duration of pentobarbital-induced sleep in mice. These derivatives also increased the γ -aminobutyric acid (GABA) level in the mouse brain after administration. This experiment was performed to determine the effect of DMP or its derivatives on the relationship between the duration of pentobarbital-induced sleep or on the interval before the appearance of picrotoxin-induced convulsion or changes in the GABA level in the mouse brain.

MATERIALS AND METHODS

Six-week-old male ddY mice (Nihon Ikagaku Dobutsu Co., Tokyo, Japan) were housed in a controlled environment ($24 \pm 1^\circ\text{C}$, $53 \pm 3\%$ humidity, lights on from 6:30 to 18:30) with food and water available *ad libitum*. DMP, aminooxyacetic acid (AOAA), and bicuculline were purchased from Sigma-Aldrich Japan Company. Valproic acid and picrotoxin

were purchased from Wako Pure Chemical Industries, Ltd. Diazepam (Horizon) was purchased from Yamanouchi Pharmaceutical Company. DMP-Cl, DMP-F and 3,6-dimethylpyrazine-2-thiol (DMP-SH) were synthesized in our laboratory according to the method described by Ohta *et al.*¹¹⁾

Effect of DMP or Its Derivatives and GABAergic Drug on the Duration of Pentobarbital-Induced Sleep

The administered dose of DMP was 50, 100, 200 or 300 mg/kg, and that of DMP-Cl, DMP-F and DMP-SH was 25, 50 or 100 mg/kg. DMP was dissolved in a 0.9% NaCl solution. DMP was administered i.p. 30 min before pentobarbital administration. DMP-Cl, DMP-F and DMP-SH were each suspended in a 0.1% methyl cellulose solution (CMC) and administered *p.o.* 1 h before pentobarbital. Thirty mg/kg of pentobarbital was injected i.p. Sleep was recognized from the disappearance of the righting reflex until its recovery. Control mice were given 0.1 ml of 0.9% NaCl (i.p.) or 1.0 ml of CMC (*p.o.*). AOAA (10 mg/kg, i.p.) was administered 6 h before pentobarbital injection. Diazepam (8.3 mg/kg, i.p.) was injected in combination with DMP.

Effect of Intracerebroventricular Injection of DMP on the Duration of Pentobarbital-Induced Sleep

Fifty, 100 and 200 $\mu\text{g}/10 \mu\text{l}$ of DMP solution was intracerebroventricularly (i.c.v.) injected just after the disappearance of the righting reflex by pentobarbital. Control mice were i.c.v. injected with 10 μl of saline alone. Sleep was recognized by the disappearance of the righting reflex until its recovery. The duration of sleep was measured between the disappearance of the righting reflex and recovery.

Effect of DMP on the Interval until the Appearance of Picrotoxin or Bicuculline-Induced Convulsion

Fifty or 100 mg/kg of DMP was injected i.p. 30 min before picrotoxin (20 mg/kg, i.p.) injection. In the case of bicuculline, bicuculline was dissolved in a 0.9% NaCl solution, and a dose of

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5 mg/kg was injected i.p. 30 min before DMP injection. The interval until the appearance of picrotoxin-induced convulsion was considered an index of the effect. Diazepam (8.3 mg/kg, i.p.) was injected 30 min before picrotoxin. Valproic acid (VPA) was dissolved in a 0.9% NaCl solution, and a dose of 200 mg/kg was injected i.p.

Effect of DMP, Aminoxyacetic Acid and Valproic Acid on GABA Level in Mouse Brain The GABA level in the mouse brain was determined by HPLC.¹²⁾ After weighing the whole brain, the brain was homogenized with 3 ml of 0.1 M perchloric acid, including β -aminoisobutyric acid (β -AIBA, 20 μ g/200 μ l 0.1 M hydrochloric acid solution) as an internal standard. The homogenate was centrifuged at 17000 rpm at 4 °C for 30 min. The supernatant was obtained and 600 μ l of 0.1 M potassium carbonate was added. Then the mixture was further centrifuged and a new supernatant was obtained. One ml of sample, 500 μ l of 0.1 M potassium carbonate and 1 ml of dancyl chloride (1.25 mg/ml) acetone solution were mixed in a test tube using a vortex mixer, then incubated at 40 °C for 30 min. After incubation, 300 μ l of 6% acetic acid solution was added and extracted with 3 ml of chloroform. The chloroform layer was evaporated to dryness by an argon stream. HPLC condition: column, Lichrosphere 100RP-18 (ϕ 5 μ m, Merck Kantoukagaku), mobile phase, H₂O, CH₃OH, CH₃CN (50 : 30 : 20 v/v), flow speed, 1.0 ml/min; and ultraviolet rays used at 254 nm. Injection volume was 5 μ l. The GABA level was calculated by the internal standard method.

Statistical Analysis Significance was determined by Dunnett's method or Tukey's test.

RESULTS

Effect of DMP or Isomers of DMP on the Duration of Pentobarbital-Induced Sleep The duration of pentobarbital-induced sleep was prolonged by the preadministration of DMP compared with that in the saline injection group. The prolongation of sleep duration was dose-dependent (Fig. 1). Figure 2 shows the effect of DMP-Cl and DMP-F compared with DMP on the duration of pentobarbital-induced sleep. Twenty-five mg/kg or 50 mg/kg of DMP-Cl and 100 mg/kg of DMP-F prolonged sleep duration. The effect of DMP-Cl or DMP-F was almost the same as that of DMP (100 mg/kg). However DMP-SH did not prolong sleep duration. Figure 3 shows the effect of DMP i.c.v. injection on pentobarbital-induced sleep duration. 200 μ g of DMP induced a greater increase in sleep duration than 50 μ g or 100 μ g of DMP injection.

Effect of AOAA and Diazepam on Pentobarbital-Induced Sleep Duration Preadministration of AOAA (10 mg/kg) or diazepam (8.3 mg/kg) induced a greater increase in sleep duration than pentobarbital alone. When DMP (100 mg/kg) or DMP-Cl (50 mg/kg) was combined with the administration of AOAA or diazepam, the duration of pentobarbital-induced sleep was more prolonged than that induced with AOAA or diazepam injection alone (Table 1). After diazepam injection, the pentobarbital-induced sleep duration increased to 6787 ± 1103 s, compared with that by pentobarbital alone (1339 ± 827 s). Then, sleep duration was prolonged to 9390 ± 2719 s by administration in combination with DMP. AOAA combined with 100 mg/kg of DMP also prolonged sleep duration compared with that induced by

Fig. 1. Effect of DMP on the Duration of Pentobarbital-Induced Sleep

DMP was administered i.p. 30 min before pentobarbital administration. 0: control, saline alone. Each column and bar represents the mean $\pm$ S.E.M. $n=5-6$. * $p<0.05$ vs. control, ** $p<0.01$ vs. control (Dunnett's test).

Fig. 2. Effect of Isomers of DMP on the Duration of Pentobarbital-Induced Sleep

DMP was administered i.p. 30 min before pentobarbital. DMP-Cl, DMP-F and DMP-SH were administered p.o. 1 h before pentobarbital. Each column and bar represents the mean $\pm$ S.E.M. $\square$, i.p. administered; $\blacksquare$, p.o. administered. $n=5-7$. * $p<0.05$ vs. control, ** $p<0.01$ vs. control (Dunnett's test).

Fig. 3. Effect of ICV injection of DMP on the Duration of Pentobarbital-Induced Sleep

Administration route: intracerebroventricular injection. Solvent: saline. Each column and bar represents the mean $\pm$ S.E.M. $n=5$. ** $p<0.01$ vs. control (Dunnett's test).

AOAA alone. DMP-Cl slightly increased sleep duration, to 3386 ± 420 s, compared with that induced by AOAA (2439 ± 348 s).

Effect of DMP on Picrotoxin- or Bicuculline-Induced Convulsion In this experiment, the effect of DMP or other drugs on the interval until the appearance of picrotoxin- or bicuculline-induced convulsion was observed. The interval

until the appearance of picrotoxin-induced convulsion was delayed by treatment with DMP combined with diazepam (Table 2). In Table 3, pretreatment with valproic acid (VPA, 200 mg/kg) slightly delayed the appearance of convulsion compared with that induced by picrotoxin alone. The combined administration of VPA and DMP further prolonged the interval until the appearance of convulsion. In the case of

bicuculline, the interval until the appearance of convulsion was significantly delayed by DMP (50, 100 mg/kg) injection. Other derivatives, DMP-Cl, DMP-F and DMP-SH, did not influence the picrotoxin-induced convulsion.

Effect of AOAA and DMP on GABA Level in Mouse Brain GABA levels in the whole brain were increased by the administration of AOAA as a transaminase inhibitor. VPA also induced increases in the GABA levels in mouse brain, to the same extent as AOAA (Table 4). Table 5 shows the effect of DMP (100 mg/kg) and DMP-Cl (200 mg/kg) on GABA levels. After DMP administration, GABA levels were significantly increased at 30 min, and the administration of DMP-Cl induced increases in GABA levels after 1 h.

DISCUSSION

In this report, we described that an adrenal gland-secreted substance⁴⁾ or alkylpyrazine derivatives, namely DMP, affected the central nervous system. DMP or its derivatives, especially DMP-Cl, affected the duration of pentobarbital-induced sleep and the interval until picrotoxin-induced convulsion or GABA levels in mouse brain. The duration of pentobarbital-induced sleep or interval until picrotoxin-induced convulsion is a basic pharmacological testing method for identifying compounds that will exhibit anxiolytic action. We selected this method because we noted in an experiment on uterine contractions that when treatment with DMP was combined with diazepam,¹³⁾ there was strong inhibition of

Table 1. Effect of DMP, AOAA and Diazepam on the Duration of Pentobarbital-Induced Sleep

Chemicals (mg/kg)	Sleeping time (s) Mean $\pm$ S.E.M.	<i>n</i>
Pento.(30)	1339 $\pm$ 827	9
+DMP(50)	1654 $\pm$ 1046	9
+DMP(100)	2206 $\pm$ 328 ^{a)}	7
+DZP(8.3)	6787 $\pm$ 1103 ^{b)}	7
+DMP-Cl(50)	2610 $\pm$ 522 ^{a)}	7
+AOAA(10)	2439 $\pm$ 348 ^{a)}	8
+DMP(50)+DZP(8.3)	9390 $\pm$ 2719 ^{c)}	7
+DMP(100)+AOAA(10)	2993 $\pm$ 33 ^{d)}	9
+DMP-Cl(50)+AOAA(10)	3386 $\pm$ 420 ^{e)}	9

Pento., pentobarbital; DMP, 2,5-dimethylpyrazine; DZP, diazepam; DMP-Cl, 2-chloro-3,6-dimethylpyrazine; AOAA, aminooxyacetic acid. *a)* $p < 0.05$ vs. Pento. (Dunnett's test). *b)* $p < 0.01$ vs. Pento. (Dunnett's test). *c)* $p < 0.05$ vs. DZP (Tukey's test). *d)* $p < 0.05$ vs. DMP (100) or vs. AOAA (Tukey's test). *e)* $p < 0.05$ vs. DMP-Cl (50) or vs. AOAA (Tukey's test).

Table 2. Effect of DMP on Picrotoxin- or Bicuculline-Induced Convulsion

Chemicals (mg/kg)	Interval until convulsion (s) Mean $\pm$ S.E.M.	<i>n</i>
Picro.(20)	378 $\pm$ 40	8
+DZP (8.3)	780 $\pm$ 90 ^{a)}	7
+DMP (100)	614 $\pm$ 99 ^{a)}	8
+DZP(8.3)+DMP(100)	1205 $\pm$ 175 ^{b)}	7
Bicu.(5)	85 $\pm$ 22	6
+DMP(50)	163 $\pm$ 18 ^{c)}	6
+DMP(100)	175 $\pm$ 92 ^{c)}	6

Picro., picrotoxin; DZP, diazepam; DMP, 2,5-dimethylpyrazine; Bicu., bicuculline. *a)* $p < 0.05$ vs. Picro. (Dunnett's test). *b)* $p < 0.05$ vs. DZP or DMP (Tukey's test). *c)* $p < 0.05$ vs. Bicu. (Dunnett's test).

Table 3. Effect of DMP or VPA on Picrotoxin-Induced Convulsion

Chemicals (mg/kg)	Interval until convulsion (s) Mean $\pm$ S.E.M.	<i>n</i>
Picro.(20)	309 $\pm$ 52	8
+DMP(100)	544 $\pm$ 79 ^{a)}	8
+VPA(200)	425 $\pm$ 67	9
+VPA(200)+DMP(100)	672 $\pm$ 81 ^{b)}	7

Picro., picrotoxin; VPA, valproic acid; DMP, 2,5-dimethylpyrazine. *a)* $p < 0.05$ vs. Picro. (Dunnett's test). *b)* $p < 0.05$ vs. VPA (Tukey's test).

Table 4. Effect of AOAA or VPA on the GABA Level in the Mouse Brain

Chemicals (mg/kg)	Time after administration				
	0	1	3	6	24 h
			(mg/g)		
AOAA (10)	0.48 $\pm$ 0.018	0.99 $\pm$ 0.114 ^{a)}	1.25 $\pm$ 0.089 ^{a)}	2.27 $\pm$ 0.264 ^{a)}	0.76 $\pm$ 0.092 ^{b)}
VPA (200)	0.44 $\pm$ 0.110	0.64 $\pm$ 0.035 ^{b)}	0.60 $\pm$ 0.125	0.62 $\pm$ 0.036 ^{b)}	0.48 $\pm$ 0.016

AOAA, aminooxyacetic acid; VPA, valproic acid. Administration route is i.p. Each value represents the mean $\pm$ S.E.M. *a)* $p < 0.01$ vs. control. *b)* $p < 0.05$ vs. control (Dunnett's test). *n* = 6–8.

Table 5. Effect of DMP or DMP-Cl on the GABA Level in the Mouse Brain

Chemicals (mg/kg)	Time after administration				
	0	0.5	1	2	3 h
			(mg/g)		
DMP (100)	0.41 $\pm$ 0.029	0.56 $\pm$ 0.043 ^{a)}	0.49 $\pm$ 0.016	0.42 $\pm$ 0.035	—
DMP-Cl (200)	0.44 $\pm$ 0.041	—	0.57 $\pm$ 0.057 ^{a)}	0.47 $\pm$ 0.037	0.47 $\pm$ 0.014

DMP, 2,5-dimethylpyrazine. Administration route is i.p. DMP-Cl, 2-chloro-3,6-dimethylpyrazine. Administration route is p.o. Each value is mean $\pm$ S.E.M. *a)* $p < 0.05$ vs. control (Dunnett's test). —: not measured. *n* = 6–8.

oxytocin or prostaglandin-induced uterine hypercontractions compared with that induced by DMP alone (unpublished observation). Accordingly, this experiment investigated DMP action on the GABAergic system in the brain. In this experiment, both the duration of pentobarbital-induced sleep and the interval until picrotoxin-induced convulsion, its prolongation of sleeping time or the appearance time of convulsion, was increased by DMP alone or in combination with diazepam. Many anti-anxiety drugs have shown the prolongation of pentobarbital-induced sleeping time and the delay of the appearance time of picrotoxin-induced convulsion. Especially, we are interested in the prolongation of pentobarbital-induced sleep duration after ICV administration of DMP, because this finding suggests that DMP may act on the central nervous system *via* the GABAergic system. Picrotoxin is known to block the action of GABA in the central nervous system and to induce convulsion. The appearance of picrotoxin-induced convulsion was delayed by diazepam, and then combined with DMP, it was even more prolonged (Table 2). Furthermore, bicuculline-induced convulsion was also delayed by DMP (Table 2). The GABA level in the brain was increased by the administration of AOAA and VPA. Both AOAA and VPA are known to inhibit the activity of GABA transaminase.¹⁴ The action of DMP combined with AOAA or VPA had a weaker effect than that of DMP with diazepam. From these combination experiments, we suggest that one mechanism of DMP may be action on a benzodiazepine receptor site. In the case of DMP, increased GABA levels were obtained at 30 min, and in the case of DMP-Cl, the increases were observed at 1 h after DMP-Cl (Table 5). Both the appearance of pharmacological action and the administration of DMP or DMP-Cl were significantly related. In this experiment, DMP-F and DMP-SH were the same or weaker than

the action of DMP. In another experiment, DMP-SH inhibited aromatase activity in rat ovary.¹⁵ Further investigation is needed of DMP derivatives with strong pharmacological actions. The data obtained from these experiments show that DMP may enhance GABAergic activity, more than inhibiting of GABA transaminase activity in the central nervous system in mice.

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