ANTICONVULSANT ACTION OF DIAZEPAM IN MICE PRETREATED WITH CAFFEINE

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Anticonvulsant activities of diazepam in mice given pentylentetrazol (PTZ), as well as effects of diazepam against potentiating activities of caffeine on these convulsions, were studied. Electroencephalographic (EEG) and electromyographic (EMG) recordings and behavioral observations were employed. First spike, clonic and tonic convulsions induced by PTZ were readily recognized from the EEG recording coupled with EMG recording. Pretreatment of mice with diazepam suppressed the incidence of clonic and tonic convulsions, but did not affect the development of first spike. These anticonvulsive effects of diazepam were also observed when PTZ-induced convulsions were potentiated with caffeine, except for clonic convulsion which was not suppressed with diazepam. The mechanisms of the antagonistic effects between diazepam and caffeine are discussed.

Keywords—diazepam; caffeine; anticonvulsant action; pentylentetrazol; electroencephalogram; electromyogram

INTRODUCTION

Pentylentetrazol (PTZ)-induced convulsion has been used for evaluation of anticonvulsant drugs. Bastian et al.1) tested some kinds of anticonvulsants in mice infused with PTZ. The mice infused with PTZ progress through four stages namely first twitch, pseudoconvulsion, persistent convulsion and death. For evaluation of anticonvulsants, they discarded first twitch recording because of the large subjective error inherent in judging the time of occurrence. However, we thought that first twitch resulted from the convulsive discharge evoked at the low threshold site of brain region attacked by PTZ, and that persistent convulsion was caused from generalization of convulsive discharge evoked with PTZ. It was thus interesting to compare the effects of anticonvulsant against the time of occurrence of first twitch and persistent convulsion. Difficulty in judging the time of occurrence of first twitch led to the use of simple EEG and EMG recordings.

One of the objects of the present work was to explore the effects of diazepam against the occurrence of four stages evoked with PTZ.

Caffeine is a central nervous system stimulant and has a convulsant action at intraperitoneal doses of 150—200 mg/kg in mice.2,3) Polec et al.4) reported a behavioral observation that caffeine antagonized the anticonvulsant activity of diazepam against PTZ-induced seizure. Another object of the present work was to make a further insight into the anticonvulsant action of diazepam in mice pretreated with caffeine from the behavioral observations and the EEG and EMG aspects.

MATERIALS AND METHODS

Animals and Drugs —The tests were made on female ICR mice weighing about 20 g. These experimental animals were housed in a cage and maintained on commercially available diet (CE-2, Clea, Tokyo) and allowed free access to food and...

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water. Drugs used were pentyleneretrazol (PTZ, Tokyo Kasei Kogyo), caffeine (Kanto Chemicals), and diazepam (Sumitomo). PTZ and caffeine were dissolved in saline. Diazepam was dissolved with 1N HCl solution and then neutralized with 1N NaOH solution, followed by dilution with saline to make the desired concentration. The concentration of the drug solution employed was such that the dose administered always represented 1 ml/100 g body weight.

**Behavioral Observation** — The assays were performed at room temperature, 24±1°C. Mice were given saline, caffeine or diazepam intraperitoneally 20 min before the administration of PTZ. When diazepam and caffeine were given concomitantly, the drug solutions were injected separately at same time. Several doses of PTZ were injected into the tail vein and the incidence of extensor tonic convulsion was examined to evaluate TD₅₀ (dose to induce 50% incidence of the tonic convulsion). TD₅₀ values and their 95% confidence limits were determined from probit-log dosage curves, each of which included four or five points. Each point consisted from 20 mice. The potency ratios and their 95% confidence limits were also calculated from probit analysis.⁵

**Electroencephalographic (EEG) and Electromyographic (EMG) Recordings** — Mice were fixed at the prone position after anesthetized with ether. For EEG recordings, the scalp was reflected and two 26-gauge needle electrodes were implaned into each cerebral hemisphere 1.5 mm lateral to the mid-sagittal suture, 1.5 mm anterior to the lambdoid suture and 1.5 mm deep from the skull. For EMG recordings, a bipolar needle electrode was positioned in the gastrocnemius muscle. After recovery from anesthesia, EEG and EMG were recorded using a multipurpose polygraph (Nihonkohden, RM-150). PTZ was infused 1 h after the implantation of electrodes according to the method of Bastian et al.¹⁹ Briefly, PTZ, 10 mg/ml in saline, was infused at the rate of 0.2 ml/min via the tail vein with a peristaltic pump (Gilson medical electric, minipulse 2). Caffeine and diazepam were injected intraperitoneally 20 min before the infusion of PTZ. For statistical evaluation, Student's t-test (two tailed) was employed. When F value was significant, Welch's method was employed.

**RESULTS**

**Behavioral Observations**

Administration of a single intravenous dose of PTZ revealed the development of tonic convolution in a dose-dependent manner (Table I). PTZ 20 mg/kg did not induce the convolution and 35 mg/kg elicited the convolution in all the mice pretreated with saline. Calculated TD₅₀ of PTZ was 24.2 mg/kg in control mice.

Pretreatment with caffeine decreased TD₅₀ of PTZ (Table I). In our experiment, mice given caffeine in doses of 50–200 mg/kg revealed no clonic and tonic convulsions, although preconvulsive symptoms such as hyperactivity and terrors were observed.

TD₅₀ of PTZ increased considerably after treatment with diazepam. To test the antagonism between diazepam and caffeine, the effect of diazepam on PTZ-induced tonic convulsion was examined in mice treated with caffeine. In this experiemnt, caffeine 200 mg/kg and diazepam 0.5 mg/kg were used. TD₅₀ of this group was higher than that of the caffeine alone treated group and lower than that of the diazepam alone treated group.

**Effects of Drugs against PTZ-Induced EEG Changes**

Fig. 1 shows typical changes of EEG and EMG following PTZ infusion. Mice infused with PTZ progress through four stages of EEG pattern: 1) first spike, 2) clonic convulsion, 3) tonic convulsion and 4) death. First spikes of EEG did not always induce concomitant changes of EMG. Following first spikes, high amplitude waves of EEG with low frequency were elicited (clonic convulsion). Afterwards, high amplitude waves of EEG with high frequency developed with the concomitant drastic changes of EMG (tonic convulsion).

The total doses of PTZ required to elicit first spike, clonic convulsion, tonic convulsion and death were calculated in mice treated with caffeine and diazepam. These values are shown in
Table II. Administrations of caffeine by itself did not induce changes of EEG and EMG. Caffeine reduced the total doses of PTZ required to elicit each wave of EEG dose-dependently.

**TABLE I. Effects of Caffeine and Diazepam on Pentylenetetrazol-Induced Convulsion in Mice**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>$TD_{50}$ (95% confidence limits) (mg/kg)</th>
<th>Potency ratio (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>50</td>
<td>24.2 (21.9—25.6)</td>
<td>1.12 (1.01—1.22)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>100</td>
<td>21.6 (19.8—23.2)</td>
<td>1.21 (1.12—1.33)</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>19.9 (17.9—21.1)</td>
<td>1.51 (1.37—1.64)</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.5</td>
<td>16.0 (14.8—16.9)</td>
<td>0.67 (0.61—0.72)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>36.5 (34.3—38.9)</td>
<td>0.45 (0.41—0.49)</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.5</td>
<td>54.2 (50.9—57.4)</td>
<td>1.09 (0.98—1.19)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>22.1 (20.4—24.0)</td>
<td>0.72 (0.66—0.79)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>200</td>
<td>1.65 (1.51—1.78)</td>
<td></td>
</tr>
</tbody>
</table>

$TD_{50}$, potency ratio and their 95% confidence limits were calculated from probit analysis.

a) compared with saline group.
b) compared with caffeine (200 mg/kg) group.
c) compared with diazepam (0.5 mg/kg) group.

**FIG. 1. Electroencephalographic (EEG) and Electromyographic (EMG) Recording Following Pentylenetetrazol Infusion in Mice**

TABLE II. Doses of Pentylentetrazol to Induce Four Patterns of EEG in Mice Treated with Caffeine and Diazepam

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Threshold dose of PTZ to induce four patterns of EEG (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>First spike</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>50</td>
<td>26.3±4.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>20.3±4.3</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>13.4±2.6</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.25</td>
<td>9.6±2.0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>25.0±3.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>23.0±2.9</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.5</td>
<td>31.5±3.6</td>
</tr>
<tr>
<td>+ Caffeine</td>
<td>50</td>
<td>25.8±5.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>19.0±6.5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10.3±4.1</td>
</tr>
</tbody>
</table>

a) Each value represents the mean ± S.E. of 5 animals.
b) p < 0.05, c) p < 0.01 as compared with saline group using the two-tailed Student's or Welch's t-test.
d) p < 0.05, e) p < 0.01 as compared with diazepam (0.5 mg/kg) group using the two-tailed Student's or Welch's t-test.
f) p < 0.05, g) p < 0.01 as compared with caffeine groups using the two-tailed Student's or Welch's t-test.

Diazepam increased the doses of PTZ required to elicit clonic convulsion, tonic convulsion and death dose-dependently. However, the development of first spikes due to PTZ was not affected by diazepam. When diazepam and caffeine were administered concomitantly, the development of four patterns of EEG change was facilitated in comparison with diazepam treated groups. In comparison with caffeine treated group, development of tonic convulsion and death were suppressed, although those of first spike and clonic convulsion were not affected.

DISCUSSION

The behavioral study demonstrated that treatment of mice with caffeine significantly increased the incidence of tonic convulsion induced by PTZ dose-dependently. Moreover, the EEG study demonstrated that in mice treated with caffeine the doses of PTZ required to elicit four patterns of EEG, namely first spike, clonic convulsion, tonic convulsion and death, decreased significantly. These EEG patterns occurred together with the characteristic patterns of EMG. Diazepam by itself did not affect the dose of PTZ to elicit first spike, whereas the doses of PTZ to induce other EEG patterns such as clonic convulsion, tonic convulsion and death were increased. In concomitant administration of diazepam and caffeine, effects of both drugs were antagonized each other on the development of tonic convulsion and death. However, the developments of first spike and clonic convulsion were not the case.

Caffeine induces convulsion at very high dose and the treatment of animals with this drug potentiates the convulsion induced with PTZ. The present work confirmed these findings. When the doses of caffeine increased in the behavioral tests, the doses of PTZ required to elicit the tonic extensor convulsion decreased. Administration of diazepam inhibited the potentiating activities of caffeine on PTZ-induced
tonic extensor convulsion.

In evaluating anticonvulsants by PTZ infusion, Bastian et al.\cite{13} stated that the time of occurrence of first twitch was hardly judged because of the large subjective error. In the present work, by the use of simple EEG and EMG recordings, the time of occurrence of first twitch was readily determined without subjective error. The first twitch was recognized as a first spike observed in EEG. Onset of tonic convulsion was recognized as high amplitude and high frequency waves of EEG with concomitant occurrence of characteristic EMG patterns.

The doses of PTZ to induce the changes of EEG patterns such as first spike, clonic convulsion and tonic convulsion were decreased considerably after treatment of mice with caffeine. Pretreatment with diazepam increased the dose of PTZ to induce clonic and tonic convulsions. However, the development of first spike was not the case. Diazepam did not affect the occurrences of first spike whether it was potentiated with caffeine or not. Benzodiazepines are reportedly effective in preventing seizure spread rather than in raising the threshold for convulsive discharge.\cite{8,9} These facts suggest that the first spike represents the convulsive discharge evoked at the low threshold sites.

A number of reports point out that benzodiazepines exert pharmacological actions by binding with the specific benzodiazepine receptors present in the brain\cite{10-12} and the pharmacological actions of benzodiazepines are related to GABAergic mechanisms which mediate the inhibitory transmission; that is, the effects of benzodiazepines are explainable with the concept that GABA transmission is a focal site of the action.\cite{12} However, correlation between this concept and anticonvulsant activity of benzodiazepines remains to be elucidated. It was reported that caffeine displaced \[^{3}H\]diazepam binding in the brain membranes.\cite{13} Peyton and Browitz\cite{14} reported that chloridiazepoxide increased calcium content in the synaptosomes from rat brain cortex and that theophylline decreased it. These results may suggest the mechanisms of the antagonistic effects between diazepam and caffeine.

Interaction between diazepam and caffeine was rather complex in PTZ-induced convulsion. Diazepam did not affect the development of first spike and clonic convulsion potentiated with caffeine. However, anticonvulsive effect of diazepam against clonic convulsion was inhibited by concomitant administration of caffeine. Diazepam and caffeine had opposite effects against the development of tonic convulsion and death. These facts may reflect that the processes of development of each convulsion were differently affected by caffeine and diazepam.

Cerebellar and cerebral levels of cyclic GMP (cGMP) and cyclic AMP (cAMP) increase after administration of convulsants such as PTZ, picrotoxin, isoniazid and bicuculline,\cite{15-18} and the increment of the brain levels of these cyclic nucleotides is suppressed by pretreatment with anticonvulsants such as diazepam, phenytoin and phenobarbital.\cite{8,15,18,20} These findings suggest that cGMP levels in the nervous system play an important role as a second messenger in the seizure activity. Diazepam and convulsants probably exert opposite effects on the mechanisms that control the level of cGMP in neurons. It may be that caffeine, a phosphodiesterase inhibitor,\cite{21} increases the levels of cyclic nucleotides in brain, the drug potentiates the convulsion, and this increment of cyclic nucleotides is antagonized with diazepam. However, the inhibitory activity of caffeine is not always specific for phosphodiesterase.\cite{22} Sattin et al.\cite{22} reported that even a convulsive dose of caffeine failed to elevate the level of cAMP in the brain and methylxanthines reduced the increase of the level of cAMP during seizures induced by electrical stimulation. Moreover, diazepam reportedly acts as an inhibitor against phosphodiesterase from the brain.\cite{28} Inhibition of phosphodiesterase is not always involved in the increase of the brain levels of cyclic nucleotides in vivo. It is one of the possible explanations that antagonism between diazepam and caffeine demonstrated in the present study may be exerted via a competitive regulation of
cGMP levels.

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REFERENCES
2) A.Satrin: Increase in the content of adenosine 3',5'-monophosphate in mouse forebrain during seizures and prevention of the increase by methylxanthines, J. Neurochem., 18, 1087—1096 (1971).
7) B.L.Welch: The significance of the difference between two means when the population variances are unequal, Biometraca, 29, 350—362 (1937).
20) C.C.Mao, A.Guidotti and E.Costa: Inhibition by diazepam of the tremor and the increase of cerebellar cGMP content elicited by harmaline, Brain Res., 83, 516—519 (1975).