Effect of Diphenylthiocarbazone (Dithizone) on Glutamate Level in Hippocampus Preparation *in Vitro* and *in Vivo*

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(Received September 11, 1989)

To assess the functional interaction between Zn^{2+} and glutamate in hippocampus, diphenylthiocarbazone (dithizone), a Zn^{2+} chelator, was used to alter the glutamate level in hippocampus *in vitro* and *in vivo*. Dithizone at the concentration of $1\ \mu\text{M}$ stimulated high K^{+} - and veratrine-induced release of $[^3\text{H}]$ glutamate both in the presence and absence of Ca^{2+} from rat hippocampal slices preloaded with $[^3\text{H}]$ glutamate without affecting the release of $[^3\text{H}]$ γ -aminobutyric acid and $[^3\text{H}]$ acetylcholine. Metal chelators other than dithizone did not evoke the $[^3\text{H}]$ glutamate release at the concentration of $10\ \mu\text{M}$. Two weeks after the intrahippocampal injection of $20\ \mu\text{g}$ of dithizone, both Zn^{2+} and glutamate levels of the hippocampus significantly decreased with no change in the levels of other metals, amino acids, monoamines and acetylcholine.

Keywords — dithizone (diphenylthiocarbazone); zinc; glutamate; release; hippocampus

Introduction

Glutamate is the most widely distributed excitatory neurotransmitter in the mammalian central nervous system.¹⁾ It has been reported that glutamate neurons are involved in several higher brain functions.²⁾ In the hippocampus, glutamate is likely a neurotransmitter candidate at the mossy fiber of granule cells.^{3,4)} Zinc (Zn^{2+}), an essential trace element, is highly concentrated in hippocampus,^{5,6)} especially in the mossy fiber axons of the dentate granule neurons.^{7,8)} It has been shown that the uptake and release of Zn^{2+} in hippocampal slices were regulated by the neuronal activity of mossy fibers.^{9,10)} Aniksztejn *et al.*¹¹⁾ reported that Zn^{2+} is released specifically from CA_3 by the stimulation of dentate gyrus paths and that colchicine blocks the release of Zn^{2+} . Zn^{2+} has an antagonistic effect on *N*-methyl-D-aspartate (NMDA) receptor,^{12,13)} a subclass of excitatory amino acid receptors. Thus, zinc is considered to be one of 'functional' substances in hippocampus.¹⁴⁾ In contrast, it is not known how Zn^{2+} affects the uptake and release of glutamate in nerve endings. In the study to clarify the functional relevance of the excitatory amino acid

neurons with Zn^{2+} in hippocampus, we found that Zn^{2+} relatively inhibited depolarization-induced release of $[^3\text{H}]$ glutamate from the preloaded hippocampal slices with $[^3\text{H}]$ glutamate (Kihara, unpublished). Furthermore, we have recently found that glutamate increases stereospecifically the dissociation of Zn^{2+} from its soluble proteins of rat hippocampus.¹⁵⁾ Together with these observations and the findings described above, we suppose that highly concentrated Zn^{2+} in hippocampus may modulate the excitatory amino acid neurotransmission. To assess the role of Zn^{2+} in functions of glutamate neurons, it is of interest to explore the condition in which hippocampal Zn^{2+} is selectively lost. Since diphenylthiocarbazone (dithizone) stains the mossy fiber terminal of CA_3 in hippocampus by its chelation of Zn^{2+} and increases the amplitude of the population spike in hippocampal slice preparations,¹⁶⁾ we examined the effect of dithizone on the release of $[^3\text{H}]$ glutamate from hippocampus. The present study shows that dithizone decreases the hippocampal glutamate preferentially both *in vitro* and *in vivo*.

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Materials and Methods

Assay of Neurotransmitter Release from Preloaded Hippocampal Slices — Release experiments by the superfusion method have been done as described previously^{17,18)} with a slight modification. Male Sprague-Dawley rats weighing 120–200 g were used throughout. Hippocampal slices (0.25 mm thick) were preincubated in Krebs-Ringer buffer (KR), which was continuously bubbled with 95% O₂–5% CO₂ at 37 °C for 20 min (about 80 mg slices/20 ml KR). KR contained in mM: NaCl, 138; KCl, 5.6; CaCl₂, 1.0; NaH₂PO₄, 1.0; NaHCO₃, 11; D-glucose, 10; *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfate (HEPES), 20 (pH 7.4). The slices were preloaded with [³H]glutamate (6 μCi/2 ml) or [³H]γ-aminobutyric acid ([³H]GABA) (4 μCi/2 ml) for 10 min. For [³H]acetylcholine ([³H]ACh) release, slices were preloaded with [³H]choline (4 μCi/2 ml) for 15 min. The preloaded slices were washed and transferred to the superfusion chambers. Slices were superfused at the rate of 2 ml/min with KR by using a peristaltic pump (Miniplus 2, Gilson). High K⁺- and veratrine-stimulations were performed by exchanging the superfusion medium to 40 mM K⁺-KR (for 16 to 26 min) or veratrine (7 μg/ml)-KR (for 16 to 18 min). Each superfusate was collected in the test tubes with a 1 min interval and their radioactivity was determined. The stimulated release was expressed as the percentage of the total radioactivity taken up by the tissues.¹⁸⁾ The present condition eliminates the contribution of re-uptake of released [³H]glutamate.¹⁸⁾ Dithizone (2.56 mg, Nakarai, Japan) was dissolved in 1 ml of ethanol, and protonated by the addition of 6 drops of concentrated NH₄OH. The volume of the stock solution was then adjusted to 10 ml with H₂O and the solution was diluted 10 to 100000-fold with high K⁺- or veratrine-KR. The final concentration of ethanol was 0.1 to 0.00001% which had no apparent effect on the release of [³H]glutamate. Aminooxyacetic acid (0.1 mM) and eserine (0.2 mM) were added to the KR for the release of [³H]GABA and [³H]ACh, respectively.

Infusion of Dithizone — Each rat was anesthetized with sodium pentobarbital (40

mg/kg, i.p.) and placed in a stereotaxic apparatus. The scalp was incised and retracted. Subjects were infused through a Hamilton microsyringe with 20 μg of dithizone in 1 μl of saline containing 0.1% ethanol in each hippocampus. Controls injected with vehicle were used. The coordinates were: 4.3 mm posterior; 3.7 mm lateral to the sagittal suture; and 3.7 mm below dura. Drug was infused over 1 min, and then the microsyringe was kept in a place for an additional 2 min before being slowly withdrawn. The scalp was then closed with sutures.

Assay of Metals, Amino Acids, Monoamines and Acetylcholine in the Hippocampus

— Hippocampi of drug treated rats were allowed microwave irradiation (7 kw for 0.9 s., Toshiba, TMW 4012, Japan). One side of the hippocampus was weighed and homogenized in 10 vols of 0.1 N HCl, and the homogenates were diluted 100-fold with 0.1 N HCl. Contents of Zn, Pb, Mn, Cu and Fe were analyzed by atomic absorption spectroscopy (Varian SPECTR AA-40 with GTA-96 graphite atomizer, U.S.A.). The other side of the hippocampus was homogenized in 10 vols of ice-cold 50 mM perchloric acid containing 0.01% ethylenediaminetetraacetic acid (EDTA), 100 μM homoserine (an internal standard for amino acid analysis), 100 μM dihydroxybenzylamine (that for monoamines) and 10 μM ethylhomocholine (that for acetylcholine) using Polytron homogenizer (Kinematica, Switzerland). Amino acid analysis was carried out by high performance liquid chromatography (HPLC) following to prelabel the amino acids with phenylisothiocyanate.¹⁹⁾ Monoamines and their metabolites were determined by HPLC with electrochemical detection (HPLC-ECD) according to the previous methods.²⁰⁾ Acetylcholine and choline were analyzed by HPLC-ECD with an immobilized enzyme reactor.²¹⁾

Data Analysis — All statistical analyses were performed utilizing the two-tailed Students *t*-test.

Results

Effect of Dithizone on Depolarization-Induced Release of [³H]Glutamate from Rat Hippocampal Slices

Fig. 1. The Effect of Dithizone on High K^+ - and Veratrine-Evoked Release of $[^3H]$ Glutamate from Slices of Rat Hippocampus

The mediums are as follows: (○), high K^+ (40 mM KCl)-KR; (●), high K^+ - Ca^{2+} -free KR; (▲), veratrine (7 μ g/ml)-KR. The procedure of the release study was described in Materials and Methods. Each point represents the mean $\pm$ S.E.M. of 4 to 5 separate experiments. a) $p < 0.05$, b) $p < 0.01$ compared with the corresponding control.

Dithizone stimulated high K^+ -induced release of $[^3H]$ glutamate in a dose-dependent manner (Fig. 1). The release was significantly increased by 145 and 175%, with 1 and 10 μ M of dithizone, respectively. Under Ca^{2+} -free condition, the release was similarly stimulated by the drug. Moreover, dithizone increased the veratrine-induced release of $[^3H]$ glutamate at the concentration of over 1 μ M. The spontaneous release of $[^3H]$ glutamate was not affected by

Fig. 2. The Effect of Dithizone on High K^+ -Induced Release of $[^3H]$ GABA and $[^3H]$ ACh from Rat Hippocampal Slices

Each point represents the mean $\pm$ S.E.M. of 4 separate experiments. Experimental details are in the Materials and Methods.

□, control; ▨, dithizone (10^{-5} M).

dithizone at the concentrations of up to 10 μ M. As shown in Table I, other metal chelators than dithizone did not stimulate the high K^+ -induced release of $[^3H]$ glutamate at the concentration of 10 μ M. In addition, both high K^+ -induced release of $[^3H]$ GABA and $[^3H]$ ACh were not affected by 10 μ M dithizone (Fig. 2).

Effect of Hippocampal Infusion of Dithizone on the Contents of Metals, Amino Acids, Monoamines and Acetylcholine

The levels of Zn^{2+} , Fe^{2+} , Cu^{2+} , Pb^{2+} and Mn^{2+} of the hippocampus 14 d after infusion of dithizone are shown in Table II. The level of Zn^{2+} was highest among the metals tested and decreased by 30% in the group pretreated with dithizone. The levels of other metals were not changed by the pretreatment with dithizone. Amino acid levels in the dithizone-pretreated hippocampus are shown in Fig. 3A. The pretreatment with dithizone resulted in a 22% decrease in the glutamate level with no change in the levels of other amino acids. Figure 3B and C show the levels of monoamines and acetylcholine in the drug-treated hippocampus, respectively. The levels of monoamines choline and acetylcholine were not changed by the drug-treatment. In addition, the amounts of monoamine metabolites such as MHPG, DOPAC, HVA, 5-HIAA were also not changed by the treatment (data not shown).

TABLE I. Effects of Chelating Agents on High K^+ -Induced Release of $[^3H]$ Glutamate from Rat Hippocampal Slices

Drug	Release of $[^3H]$ glutamate (%)
Control	4.86 $\pm$ 0.67
Dithizone (Diphenylthiocarbazone)	9.28 $\pm$ 1.06 a)
Diethyldithiocarbamate	5.36 $\pm$ 1.38
<i>o</i> -Phenanthroline	5.24 $\pm$ 1.11
Tetraethylenepentamine	5.51 $\pm$ 0.72

Each of the drugs (10 μ M) are added in the medium as described in Materials and Methods. Each value represents the mean $\pm$ S.E.M. of 4 separate experiments. a) $p < 0.05$, compared with control.

TABLE II. Changes in Metal Contents of Hippocampus by Intrahippocampal Injection of Dithizone

Condition	Metal content ($\mu\text{g/g}$ wet weight)				
	Zn	Fe	Cu	Pb	Mn
Sham	13.97 ± 0.25	5.74 ± 0.19	2.20 ± 0.27	2.03 ± 0.20	0.51 ± 0.05
Vehicle	14.40 ± 0.23	6.92 ± 1.17	1.71 ± 0.07	1.92 ± 0.07	0.51 ± 0.03
Dithizone (Diphenylthiocarbazone)	$10.24 \pm 0.57^a)$	5.55 ± 0.18	1.73 ± 0.21	1.95 ± 0.12	0.55 ± 0.05

Dithizone ($20 \mu\text{g}$ in $1 \mu\text{l}$) was infused into rat hippocampus bilaterally 2 weeks before sacrifice. Values are the mean $\pm$ S.E.M. of 6 separate experiments. *a)* $p < 0.05$, compared with vehicle.

Discussion

Two findings of special interest are apparent from the present study. One is that dithizone caused a selective release of [^3H]glutamate from the hippocampal slices preloaded with [^3H]glutamate, and the other is that the content of en-

dogenous glutamate in hippocampus was preferentially decreased by an intrahippocampal pre-injection of dithizone with a concomitant decrease in the Zn^{2+} level. Dithizone and certain other chelating agents bind Zn^{2+} and thus affect neuronal functions.^{22,23)} Systematically administered dithizone significantly decreases the peak

Fig. 3. The Levels of Amino Acids(A), Monoamines(B), and Acetylcholine(C) in Rat Hippocampus after Intrahippocampal Injection of Dithizone

The experimental procedure for an infusion of dithizone is as the same as that of Table II. Each point represents the mean $\pm$ S.E.M. of 6 separate experiments. *a)* $p < 0.05$ compared with vehicle.

□, sham; ▨, vehicle; ▩, dithizone ($20 \mu\text{g} \times 2, 1\text{h}$).

frequency of synchronous rhythmic slow activity in hippocampal EEG.²⁴⁾ Dollar and Crawford¹⁶⁾ reported that low concentrations of dithizone (4–50 μM) increase the amplitude of the population spike following the stimulation of the dentate gyrus of the hippocampus possibly through a direct action on neuronal membranes. This would be explained by the present finding of which dithizone enhanced the depolarization-induced release of glutamate. D-Aspartate exchanges across the synaptic membranes on the same carrier as L-glutamate²⁵⁾ and thus exchange glutamate out of the cytoplasm in Ca^{2+} -independent manner.²⁶⁾ However, dithizone does enhance the release of [^3H]glutamate by different mechanisms than D-aspartate does, because it did not influence the spontaneous release of [^3H]glutamate. Furthermore, the specificity of the effect of dithizone on glutamate release eliminates the possibility of its action being related to non-specific membrane effects. Dithizone is known to influence not only the histochemical staining of zinc-containing neural elements,²⁷⁾ but also the concentration of hippocampal zinc.^{28,29)} Consistent with the previous observations, the intrahippocampal application of dithizone reduced specifically the content of Zn^{2+} in the hippocampus. At present, it is not clear whether dithizone accelerates the release of [^3H]glutamate only by chelating Zn^{2+} in the neurons. Aniksztejn *et al.*¹¹⁾ reported that about 30 μM of Zn^{2+} is released in the area of CA_3 by K^+ -pulse stimulation in the vicinity of the mossy fibers. We have recently found that 10 μM Zn^{2+} inhibited the release of [^3H]glutamate from the hippocampal slices without affecting the release of [^3H]GABA and [^3H]ACh (Kihara, unpublished). Based on these observations, the stimulation by dithizone on [^3H]glutamate release may be explained by a chelation of extracellular Zn^{2+} which is released by depolarization. However, the effect of dithizone on [^3H]glutamate release was observed even at the concentration of 1 μM . It is suggested that dithizone chelates intracellular Zn^{2+} and that consequently enhanced the release of glutamate.

Recently, it has been reported that Zn^{2+} inhibits NMDA receptor-mediated channels.^{12,13)} Glutamate enhances the dissociation of Zn^{2+}

from soluble proteins of rat hippocampus.¹⁵⁾ The present study shows that dithizone causes selective loss of Zn^{2+} and glutamate in the hippocampus. It is not known whether the *in vivo* effect of dithizone could be explained by its *in vitro* effect, because the decrease in glutamate level was rather small in comparison with the *in vitro* effect of the drug. We have not yet examined the sub-regional and subcellular distribution of glutamate in the hippocampus after application of dithizone. However, it may be expected that the reduction of glutamate level after the drug treatment is a reflection of a change in nerve terminals by the following reason: the *in vitro* experiment of [^3H]glutamate release suggests that dithizone depletes [^3H]glutamate from the nerve terminals of glutamate neurons, because the preloading of [^3H]glutamate by a high affinity uptake system is restricted within nerve terminals. As described above, Zn^{2+} has inhibitory effects of glutamatergic neurotransmission. In this connection, the present study also suggests an inhibitory role of endogenous Zn^{2+} in glutamate neurons.

Acknowledgments This work was supported by a grant from the Ministry of Education, Science, Culture of Japan.

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