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Adenosine transport inhibitors enhance high K^+-evoked taurine release from rat hippocampus

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Abstract

We examined the effects of Ca^{2+}-free medium containing 20 mM Mg^{2+}, a non-selective adenosine receptor antagonist, theophylline, and adenosine transport inhibitors, dipyridamole and nitrobenzylthioinosine, on high K^+-evoked spreading depression, glutamate, and taurine release from the rat hippocampus using brain microdialysis. High K^+ alone perfusion evoked spreading depression and increased glutamate release followed by taurine efflux. Perfusion of Ca^{2+}-free medium with high K^+ never evoked spreading depression and decreased the high K^+-evoked taurine release. Perfusion of theophylline (1 mM) increased the occurrence of high K^+-evoked spreading depression and glutamate release, but did not modify taurine release. In contrast, simultaneous perfusion of dipyridamole (100 μM) and nitrobenzylthioinosine (50 μM) reduced the occurrence of spreading depression and the high K^+-evoked glutamate release, but enhanced significantly the taurine efflux. These findings suggest that endogenous taurine with adenosine may have neuroprotective actions against high K^+-evoked glutamate release and spreading depression in the rat hippocampus, in addition to its osmoregulatory action.

Keywords: Taurine; Adenosine transport inhibitor; Adenosine; Spreading depression; NMDA receptor; Hippocampus

1. Introduction

Spreading depression (Leao, 1944) is known to be associated with neuropathological conditions such as cerebral hypoxia/ischemia, concussion, and migrane in humans (Lauritzen, 1987; Somjen, 1988). Spreading depression is associated with a transient negative potential shift and a marked increase in extracellular K^+ (Bures et al., 1974; Nicholson and Kraig, 1981). Glutamate release may be involved in spreading depression (Van Harreveld and Kooiman, 1965). During spreading depression, K^+ flows out of neurons and glial cells and Na^+, Cl^-, Ca^{2+}, and water, conversely, flow into these cells, resulting in cell swelling (Somjen et al., 1992). Activation of NMDA receptors by endogenous release of glutamate plays a crucial role in triggering spreading depression (Gorelova et al., 1987; Hernandez-Caceres et al., 1987; Marrannes et al., 1988). The intracellular Ca^{2+} concentration increases during spreading depression (Somjen and Aitken, 1984).

Adenosine is a neuromodulator (Dunwiddie, 1985; Stone, 1991) that presynaptically blocks the release of glutamate (Corradetti et al., 1984) and postsynaptically hyperpolarizes pyramidal cells in the hippocampal CA1 region via adenosine A_1 receptors (Siggins and Schubert, 1981). We have recently demonstrated, by microdialysis, that the increase in endogenous adenosine elicited by adenosine transport inhibitors exerts an inhibitory influence, through the adenosine A_1 receptor, on the high K^+-evoked glutamate release and spreading depression in the rat hippocampus (Kaku et al., 1994).

Extracellular taurine levels in the hippocampus are increased by application of excitatory amino acids (Lehmann et al., 1984; Menendez et al., 1990; Magnusson et al., 1991) and by ischemia (Benveniste et al., 1984; Hagberg et al., 1985). Taurine is known to be involved in osmoregulation (Wade et al., 1988; Menendez et al., 1990; Huxtable, 1992; Vitarella et al., 1994). Moreover, taurine has protective effects against epilepsy (Laird and Huxtable,

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1978; Van Gelder, 1978; Durelli and Mutani, 1983; Oja and Kontro, 1983; Toth et al., 1983), hypoxia/ischemia (Schurr et al., 1987; Malcangio et al., 1989), and excitotoxicity (French et al., 1986; Magnusson et al., 1991). NMDA receptor activation is involved in these conditions (Durelli and Mutani, 1983; Oja and Kontro, 1983; Toth et al., 1983; French et al., 1986; Schurr et al., 1987). Taurine inhibits high K^+-evoked release of glutamate and γ-aminobutyric acid (GABA) from rat cortical synaptosomes (Kamisaki et al., 1993).

The aim of the present study was to examine whether or not taurine release is associated with adenosine release. To this end we studied the effects of adenosine transport inhibitors, dipyridamole and nitrobenzylthioinosine, on the high K^+-evoked taurine release and concomitant spreading depression in the rat hippocampus, using microdialysis. We used dipyridamole and nitrobenzylthioinosine together to increase endogenous adenosine effectively. We also examined the effects of a non-selective adenosine receptor antagonist, theophylline, on the high K^+-evoked taurine release to compare its effects with those of adenosine transport inhibitors.

2. Materials and methods

Subjects were male Wistar rats weighing 270–330 g. All experimental animal procedures were carried out according to 'Guiding Principles for Care and Use of Animals in the Field of Physiological Sciences' recommended by The Physiological Society of Japan. Methods of preparing animals, including implantation of stimulating and recording electrodes, were about the same as those reported previously (Kaku et al., 1994). Therefore, essential points are noted here. Rats were anesthetized with urethane (1.5 g/kg, i.p.). To record population spike responses and background EEG from the hippocampal CA1 region, stimulating bipolar stainless steel electrodes and recording glass-microelectrodes were implanted in the dorsal hippocampus. A dialysis probe (CMA/10, Carnegie Medicine) was stereotaxically inserted into the dorsal hippocampus. The probe was about 0.5 mm in diameter and had a 1-mm long membrane at the tip.

The probe was perfused at a rate of 2.0 μl/min with artificial cerebrospinal fluid (ACSF) (composition in mM: NaCl 132.8; KCl 3.0; $CaCl_2$ 2.0; $MgCl_2$ 0.7; $NaHCO_3$ 24.6; urea 6.7; glucose 3.7) for an equilibration period of 120 min, using a microinfusion pump (CMA/100, Carnegie Medicine). Drops of perfusate (20 μl) were collected every 10 min. Fractions were frozen and stored at −80°C until analysis.

The experiments were performed with the following 4 groups: (1) high (75 mM) K^+ alone group ($n = 7$) as control group, (2) Ca^{2+}-free medium with 20 mM Mg^{2+} group (Ca^{2+}-free medium group, $n = 4$), (3) the non-selective adenosine receptor antagonist, theophylline (1 mM) group ($n = 5$), and (4) adenosine transport inhibitors, dipyridamole (100 μM) plus nitrobenzylthioinosine (50 μM) group ($n = 6$). After a 120-min stabilization period, all groups underwent the basically identical following sequence of perfusion: (1) ACSF for 40 min, (2) 75 mM K^+ alone or 75 mM K^+ plus drugs for 40 min, and (3) ACSF again for 40 min. However, Ca^{2+}-free medium or theophylline was applied 20 min before perfusion of high K^+ for 40 min and perfused in total for 60 min. The extracellular K^+ concentration nearby the probe (1-mm long membrane) used in the present experiment was estimated as about 25% of the 75 mM K^+ concentration in the perfusate. This value was obtained by measuring K^+ concentrations in inlet and outlet tubes of the probes by atomic absorption spectroscopy.

Concentrations of glutamate and taurine were determined by reverse-phase liquid chromatography using a fluorescence detector after precolumn derivatization with *o*-phthaldialdehyde. An MA-5ODS (EICOM) column was used. The mobile phase was 75% phosphate buffer (pH 6.0) with 10% methanol and 15% tetrahydrofuran and was pumped at a flow rate of 0.6 ml/min. The amounts of glutamate and taurine were determined by measuring the peak area with reference to external standards. The percent recovery of glutamate and taurine in vitro from the probes (1 mm) was tested as follows. They were placed in a bath medium containing ACSF with glutamate or taurine of 1.0–5.0 μM (maintained at 37°C) and then perfused at 2.0 μl/min with ACSF. The in vitro mean recovery was: 8.3 ± 0.7% ($n = 5$) for glutamate; 12.3 ± 0.5% ($n = 5$) for taurine.

The Schaffer-commissural fibers were stimulated with pulses (100 μs, 500–700 μA) at a frequency of 0.1 Hz, using an electronic stimulator (Nihon Kohden, SEN-3201). Population spike responses were amplified at a 0.3-s time constant through an AC amplifier (Nihon Kohden, AVB-10), visualized on a memory oscilloscope (Nihon Kohden, VC-10), and saved on a cassette data recorder (Nihon Kohden, RMG-5104).

At the end of each experiment, a dye mark was made by passing a negative current (10 μA) for 1 min through the microelectrode to verify the recording site of the population spike response. The brains were dissected, sliced into 60-μm sections, and stained with cresyl violet. Placement of the dialysis probe and electrodes was histologically verified.

Theophylline, dipyridamole, and nitrobenzylthioinosine were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). All standards and reagents used for HPLC analysis were supplied by Bioanalytical Systems. Dipyridamole and nitrobenzylthioinosine were dissolved in dimethyl sulphoxide and then diluted to 100 μM and 50 μM, respectively, in ACSF containing 75 mM K^+. Dimethyl sulphoxide alone did not affect glutamate and taurine release.

Results are presented as the means ± S.E.M. Glutamate

or taurine release is expressed as a percentage, taking the mean basal amount released during ACSF perfusion for 20 or 40 min before application of high K^+ or any drug as 100% (Figs. 1 and 2). Total evoked taurine release was the cumulative amount of taurine released during a 60-min period following the onset of high K^+ perfusion (Fig. 3). This was obtained by subtracting the amount of taurine released before any drug perfusion from that released during the 60-min period following the onset of high K^+ perfusion. Taurine release is expressed as a percentage, taking the mean total net amount of taurine released by high K^+ alone as 100%. Statistical analysis of the data for multiple comparisons was performed by a one-way analysis of variance (ANOVA) and a mixed type ANOVA with repeated measures and post-hoc tests (Fisher's protected least significant difference). For single comparisons, the significance of differences between means was determined by Student's two-tailed *t*-tests. Differences were considered significant at $P < 0.05$.

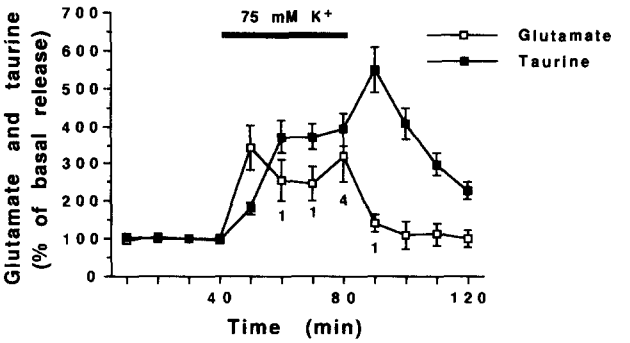

Fig. 1. Time course of high K^+-evoked release of glutamate and taurine in the high K^+ alone group. Each point with a vertical bar represents the mean ± S.E.M. amount of glutamate or taurine released for 7 experiments and is expressed as a percentage, taking the mean basal taurine release before any drug application as 100%. The occurrence of high K^+-evoked spreading depression during the sampling period is presented under each glutamate value. Note that the peak levels of glutamate release always preceded those of taurine release by one sampling period (10 min) and that the second peak of glutamate release was associated with a high incidence of spreading depression.

3. Results

Spreading depression was usually evoked once or twice by a 40-min period of perfusion with high K^+ alone. Perfusion with Ca^{2+}-free medium alone reduced population spike amplitudes and even addition of high K^+ to the perfusate never evoked spreading depression. The non-selective adenosine receptor antagonist, theophylline, enhanced the occurrence of high K^+-evoked spreading depression and abolished the population spikes. In contrast, the adenosine transport inhibitors, dipyridamole and nitrobenzylthioinosine, reduced the occurrence of spreading depression, but did not change the population spike amplitude in most cases.

3.1. Time course of high K^+-evoked glutamate and taurine release

The basal levels of glutamate and taurine were 0.19 ± 0.03 μM ($n = 7$) and 0.73 ± 0.10 μM ($n = 7$), respectively. Fig. 1 shows the time course of high K^+-evoked glutamate and taurine release in the high K^+ alone group. The amount of glutamate released increased 3.5- and 3.0-fold, 10 and 40 min after the onset of high K^+ perfusion, respectively. The second peak of glutamate release was closely associated with the occurrence of spreading depression. In contrast, the amount of taurine released increased 3.7-fold 20 min after the onset of high K^+ perfusion and 5.5-fold 10 min after the onset of ACSF reperfusion and then decreased gradually. A one-way repeated measures ANOVA, to compare the basal with the post-high K^+ taurine levels in the hippocampus, revealed a significant main effect [$F(8,48) = 34.888$, $P = 0.0001$]. Post-hoc comparisons of taurine levels following high K^+ perfusion with basal values (4 samples preceding high K^+ perfusion) indicated there was a significant elevation in taurine release for 80 min or longer. The peak levels of high K^+-evoked glutamate release always preceded those of taurine release by one sampling period (10 min).

3.2. Effects of Ca^{2+}-free medium on high K^+-evoked taurine release

In order to examine whether neurons or glial cells might have been responsible for high K^+-evoked taurine release, the effects of Ca^{2+}-free medium (Ca^{2+}-free ACSF containing 20 mM Mg^{2+}) on the evoked release were tested. Perfusion of the Ca^{2+}-free medium alone reduced slightly the basal release of taurine. Application of high K^+ to the Ca^{2+}-free medium increased significantly the taurine release (Fig. 2), but never evoked spreading depression. After reperfusion of ACSF, the taurine level returned gradually to the basal level. The high K^+-evoked taurine release over basal release was high in the Ca^{2+}-containing medium (Fig. 1) and low in the Ca^{2+}-free medium (Fig. 2). A one-way repeated measures ANOVA, to compare the basal taurine level after ACSF perfusion with taurine levels after Ca^{2+}-free medium perfusion, revealed a significant main effect [$F(10,30) = 12.783$, $P = 0.0001$]. Post-hoc comparisons of taurine levels following perfusion with Ca^{2+}-free medium with basal values (samples preceding Ca^{2+}-free medium perfusion) indicated significant reductions in taurine release.

3.3. Effects of theophylline on high K^+-evoked taurine release

We examined the effects of the non-selective adenosine receptor antagonist, theophylline, on high K^+-evoked taurine release, to compare its effects with those of the

adenosine transport inhibitors. Perfusion of theophylline (1 mM) alone did not affect the basal release of taurine. However, addition of high K^+ to the medium containing theophylline increased taurine release 4-fold (Fig. 2). After reperfusion of ACSF, the taurine release decreased gradually to the basal level. A one-way repeated measures ANOVA revealed a significant main effect [$F(10,40) = 20.149$, $P = 0.0001$]. Post-hoc comparisons of taurine levels following theophylline and high K^+ perfusion with basal levels indicated significant elevations in taurine release for up to 80 min or longer.

3.4. Effects of dipyridamole plus nitrobenzylthioinosine on high K^+-evoked taurine release

Perfusion of dipyridamole (100 μM) plus nitrobenzylthioinosine (50 μM) alone did not exert any appreciable effect on the taurine levels in the dialysates (data not shown). Simultaneous perfusion of dipyridamole plus nitrobenzylthioinosine with high K^+ enhanced gradually the taurine release 5- to 6-fold (Fig. 2). After reperfusion of ACSF, the taurine release decreased slowly to the basal level. A one-way repeated measures ANOVA revealed a significant main effect [$F(8,40) = 33.358$, $P = 0.0001$]. Post-hoc comparisons of taurine levels following dipyridamole plus nitrobenzylthioinosine and high K^+ perfusion with basal levels indicated significant elevations in taurine release level for up to 80 min or longer.

3.5. Comparison of effects of Ca^{2+}-free medium, theophylline, and dipyridamole plus nitrobenzylthioinosine on high K^+-evoked taurine release

The increase in high K^+-evoked taurine release in the dipyridamole plus nitrobenzylthioinosine group was the largest among the 4 groups, while that in the Ca^{2+}-free medium group was the smallest (Fig. 2). A mixed type ANOVA with repeated measures design was carried out to analyse the time course of taurine release. The main effects of both group and time were significant [$F(3,18) = 6.784$, $P = 0.003$ and $F(11,98) = 96.295$, $P = 0.0001$, respectively]. The interaction between group and time was also significant [$F(33,198) = 5.214$, $P = 0.0001$]. Fisher's protected least significant difference post-hoc test showed significant group differences ($P < 0.05$) between the high K^+ alone group and the Ca^{2+}-free medium group and between the high K^+ alone group and the dipyridamole plus nitrobenzylthioinosine group. However, a significant difference ($P < 0.05$) between the high K^+ alone group and the theophylline group could not be found by Fisher's protected least significant difference post-hoc test.

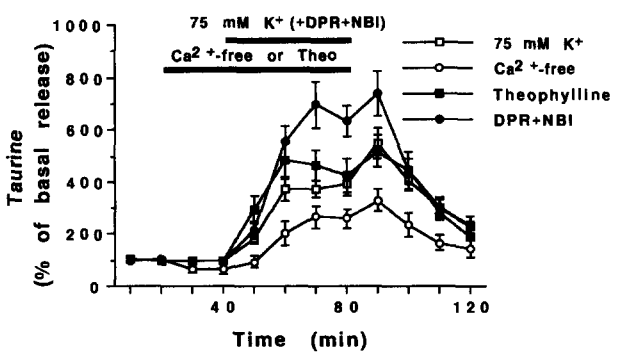

Fig. 2. Time course of taurine release evoked by perfusion of high K^+ alone, Ca^{2+}-free medium, theophylline, and dipyridamole (DPR) plus nitrobenzylthioinosine (NBI). Each point with a vertical bar represents the mean ± S.E.M. amount of taurine released for 4–7 experiments and is expressed as a percentage, taking the mean basal taurine release before any drug application as 100%. Statistical analysis was done with a mixed type ANOVA with repeated measures design. Fisher's protected least significant difference post-hoc test showed significant group differences ($P < 0.05$) between the high K^+ alone group and the Ca^{2+}-free medium group and between the high K^+ alone group and the dipyridamole plus nitrobenzylthioinosine group. See text for further details.

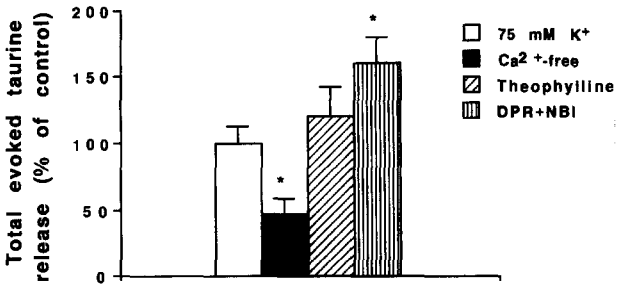

Fig. 3. Effects of perfusion of Ca^{2+}-free medium, theophylline, and dipyridamole (DPR) plus nitrobenzylthioinosine (NBI) on the total net amount of high K^+-evoked taurine release. The total net amount of taurine was obtained by subtracting the basal amount of taurine released before any drug application from that released during the 60-min period following the start of high K^+ perfusion. Each column with a vertical bar represents the mean ± S.E.M. of 6 samples obtained from 4–7 animals and is expressed as a percentage, taking the mean total net amount of high K^+-evoked taurine release in the high K^+ alone group as 100%. Note that the high K^+-evoked taurine release in the Ca^{2+}-free medium group was the lowest among the 4 groups, whereas the taurine release in the dipyridamole plus nitrobenzylthioinosine group was the highest. * $P < 0.05$, Student's two-tailed t-test.

We also estimated the total net amount of taurine released during the 60-min period following the onset of high K^+ perfusion, as shown in Fig. 3 (see Materials and methods). Perfusion of Ca^{2+}-free medium reduced significantly the total net amount of high K^+-evoked taurine release by 53% ($P < 0.05$). In contrast, perfusion of dipyridamole plus nitrobenzylthioinosine enhanced it significantly by 61% ($P < 0.05$). The effect of theophylline (21%) was not statistically significant.

4. Discussion

In the present study, we examined the effects of adenosine transport inhibitors, dipyridamole plus nitrobenzylthioinosine, on high K^+-evoked taurine release, using microdialysis. The major findings of the present study were:

(1) the peak level of the high K^+-evoked release of glutamate always preceded that of taurine; (2) the non-selective adenosine receptor antagonist, theophylline, did not modify the high K^+-evoked taurine release; and (3) adenosine transport inhibitors enhanced markedly the high K^+-evoked taurine release. The amount of taurine released by high K^+ was ranked as follows: dipyridamole plus nitrobenzylthioinosine > theophylline > high K^+ alone > Ca^{2+}-free medium.

The present in vivo finding that the peak level of high K^+-evoked release of glutamate always preceded that of taurine in the hippocampus could be explained as follows. Glutamate is released from presynaptic terminals of glutamatergic neurons and from glial cells by high K^+-induced depolarization. Following activation of NMDA receptors by the released glutamate, taurine is released from cells. The amino acid is also released from swollen cells to regulate the high K^+-induced osmotic imbalance. High K^+ evokes glutamate release followed by taurine efflux. A similar salient feature of high K^+-evoked release of glutamate and taurine was observed in in vivo striatum (Girault et al., 1986) and cultured brain tissues (Holopainen et al., 1989; Schousboe and Pasantes-Morales, 1989). This explanation is in line with the following reports. Activation of NMDA receptors by glutamate receptor agonists evokes taurine release (Lehmann et al., 1984; Menendez et al., 1990, 1993). Taurine is primarily released by concentrations of glutamate receptor agonists that cause damage to neurons (Magnusson et al., 1991). Systemic administration as well as local application of NMDA into the brain increases extracellular taurine levels (Shibanoki et al., 1993). Endogenous taurine is released from cultured cerebellar neurons and astrocytes by high K^+ stimulation and the maximal taurine efflux occurs 5–7 min after the maximal K^+ concentration (Philibert et al., 1989a, Philibert et al., 1989b). High K^+ also induces cell swelling (Kamino et al., 1973; Walz, 1987) followed by taurine release (Solis et al., 1986; Pasantes-Morales and Schousboe, 1989; Kimelberg et al., 1990; Martin del Rio et al., 1990; Pasantes-Morales et al., 1990), which is involved in osmoregulation (Wade et al., 1988; Menendez et al., 1990; Huxtable, 1992; Vitarella et al., 1994). Moreover, activation of adenosine receptors by the released glutamate may stimulate taurine release by increasing intracellular cAMP levels in astroglial cells (Madelian et al., 1988). These findings are consistent with the present result that taurine release actually preceded glutamate release.

The present in vivo results also showed that the high K^+-evoked taurine release from the rat hippocampus is in part Ca^{2+}-dependent, although blockade of the NMDA receptor-controlled channels by the high concentration of Mg^{2+} in the perfusate may be essential for this process. The present finding is in agreement with the in vitro finding of Oja et al. (1985) that both Ca^{2+}-dependent and independent release of taurine may originate from neurons and glial cells.

Dipyridamole plus nitrobenzylthioinosine reduced the high K^+-evoked glutamate release and the occurrence of spreading depression (Kaku et al., 1994) but enhanced the taurine release in the present study. In contrast, theophylline enhanced the high K^+-evoked glutamate release and the occurrence of spreading depression (Kaku et al., 1994), but not the taurine release. Theophylline thus antagonized the effects of dipyridamole plus nitrobenzylthioinosine on the high K^+-evoked glutamate release and spreading depression, but not on the taurine release. Theophylline would increase endogenous adenosine levels via NMDA receptor activation, because it enhances high K^+-evoked glutamate release (Kaku et al., 1994). Since theophylline blocks adenosine receptors, the probably increased level of endogenous adenosine may not have activated adenosine receptors. This could be the reason why theophylline did not modify the high K^+-evoked taurine release in the present study.

At present, we do not know why dipyridamole plus nitrobenzylthioinosine reduced the high K^+-evoked spreading depression but enhanced markedly the taurine release, as compared to the effects elicited by perfusion with high K^+ alone. Dipyridamole plus nitrobenzylthioinosine enhance high K^+-evoked adenosine release (Kaku et al., 1994). The increase in endogenous adenosine may also be involved in the increased release of taurine through adenosine receptor activation (Madelian et al., 1988). Moreover, an adenosine A_1 receptor agonist, phenylisopropylthioinosine, reduces high K^+-evoked glutamate release, but not taurine efflux (Hada et al., 1995). This indicates that the enhancement of high K^+-evoked taurine release by adenosine transport inhibitors may be related to mechanisms other than adenosine A_1 receptor-mediated mechanisms.

We demonstrated in the present study that high K^+-evoked taurine release is enhanced by adenosine transport inhibitors which reduce spreading depression (Kaku et al., 1994). Taurine acts on presynaptic terminals to inhibit K^+-evoked glutamate release from rat cortical synaptosomes (Kamisaki et al., 1993). Taurine also hyperpolarizes hippocampal neurons (Zeise, 1985; Taber et al., 1986). Taurine could protect neuronal and glial cells from Ca^{2+} toxicity by maintaining Ca^{2+} homeostasis (Huxtable, 1992). Taurine, together with endogenous adenosine as reported previously (Kaku et al., 1994), is likely to be involved in the reduced occurrence of spreading depression in the hippocampus. The idea is supported by previous reports that taurine has protective effects against epilepsy (Durelli and Mutani, 1983; Oja and Kontro, 1983; Toth et al., 1983), hypoxia/ischemia (Schurr et al., 1987), and excitotoxicity (French et al., 1986) through NMDA receptor activation. Thus, taurine probably has neuroprotective actions under experimental and pathological conditions such as high K^+ and ischemia.

In conclusion, the present findings demonstrated that dipyridamole plus nitrobenzylthioinosine reduces the occurrence of high K^+-evoked spreading depression and

enhances high K^+-evoked taurine release from the hippocampus, and also suggested that endogenous taurine with adenosine has neuroprotective actions against high K^+-evoked spreading depression, in addition to its osmoregulatory action. The mechanisms underlying the neuroprotective cooperation between adenosine and taurine remain to be solved.

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