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# Sodium nitroprusside-induced seizures and adenosine release in rat hippocampus

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**Abstract**

In the present study, we examined the effects of nitric oxide (NO)-related compounds, i.e. sodium nitroprusside (NO donor), diethyldithiocarbamate (NO trapper) and dithiothreitol (superoxide radical scavenger) on release of aspartate and adenosine from rat hippocampus using electrophysiological and microdialysis methods. Perfusion with 0.05 or 0.5 mM sodium nitroprusside significantly reduced high $K^+$-evoked release of aspartate during high $K^+$ perfusion. Perfusion with 0.5 mM sodium nitroprusside always induced seizures and significantly increased release of aspartate and adenosine during washout of sodium nitroprusside. Diethyldithiocarbamate (5 mM) reversed the effects of sodium nitroprusside. Dithiothreitol (1 mM) significantly reduced the increase in adenosine release by sodium nitroprusside. These findings indicate that adenosine release is closely related to development of seizures, which are triggered by an increase in both NO itself and in part peroxynitrite, which results in reaction with superoxide radicals. © 2001 Published by Elsevier Science B.V.

*Keywords:* Seizure; Adenosine; Nitric oxide (NO); Nitric oxide (NO) donor; Sodium nitroprusside; Microdialysis; Hippocampus

## 1. Introduction

Nitric oxide (NO) has been known to regulate many cerebral physiological processes including circulation and neuronal transmission. NO may also contribute to neurotoxicity under pathological conditions such as cerebral ischemia and epilepsy. In in vitro study, NO has been shown to mediate *N*-methyl-D-aspartate (NMDA) neurotoxicity in rat hippocampal slices (Izumi et al., 1992). In in vivo study, intrahippocampal microinjection of an NO donor, sodium nitroprusside, in the rat has been shown to result in a marked degeneration of the hippocampal formation (Loiacono and Beart, 1992). Furthermore, motor and electrographical seizures have been shown after microinjection of sodium nitroprusside into the deep prepiriform cortex (De Sarro et al., 1993) and hippocampus (Cuajungco and Lees, 1998).

Adenosine has a variety of actions in the nervous system, including suppression of spontaneous neuronal firing, inhibition of synaptic transmission and transmitter release (for review, Brundege and Dunwiddie, 1997). In addition, adenosine is released during occurrence of seizures and also acts as an endogenous anticonvulsive agent (for review, Dunwiddie, 1999). Thus, both NO and adenosine are involved in neuronal functions, including neuronal transmission, neuroprotection, neurotoxicity and cerebral blood flow. Furthermore, it has recently been shown that NO modulates basal adenosine release from in vivo striatum (Fischer et al., 1995) and hippocampal slices (Fallahi et al., 1996). On the other hand, adenosine release has not been influenced by sodium nitroprusside in rat cortical slices (Craig and White, 1993). Thus, modulatory effects of NO on adenosine release remains controversial.

In our previous study, an NO trapper, diethyldithiocarbamate, completely abolished seizures induced by intrahippocampal perfusion with sodium nitroprusside and a su-

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peroxide radical scavenger, dithiothreitol, reduced the frequency of the seizures (Hada et al., 2000). In the present study, we examined the effects of sodium nitroprusside, diethyldithiocarbamate and dithiothreitol on release of aspartate and adenosine in rat hippocampus in vivo using electrophysiological and microdialysis methods.

## 2. Materials and methods

### 2.1. Animals and surgery

All experimental animal procedures were carried out according to the Guiding Principles for Care and Use of Animals in the Field of Physiological Sciences as recommended by The Physiological Society of Japan. Adult male Wistar rats, weighing 270–330 g, were anesthetized with urethane (1.2 g/kg, i.p.) and fixed in a stereotaxic frame. Body temperature was maintained at 37–38°C by a heating pad. A microdialysis probe (CMA/10, CMA/Microdialysis, outer diameter 0.5 mm, dialysis membrane length of 2 mm; cut-off 20 kDa) was inclined 20° posteriorly in a parasagittal plane. The tip of the probe was introduced in the dorsal hippocampus (5.5 mm caudal to the bregma, 3.5 mm lateral to the midline and 4.0 mm below the cortical surface), according to the atlas of Paxinos and Watson (1982). For stimulating the Schaffer collateral–commissural pathway, a concentric bipolar stimulating electrode was inserted through the cortex at an angle of 20° inclined anteriorly in a parasagittal plane and fixed stereotaxically into the stratum radiatum of the CA3 region (2.5 mm caudal to the bregma, 2.5 mm lateral to the midline and 3.3 mm below the cortical surface). A glass-microelectrode was inserted into the stratum pyramidale of the CA1 region (0.5 mm lateral to the microdialysis probe) to record population spikes and background electroencephalogram (EEG). The recording electrode was fixed in the place where the maximal amplitude of population spikes was obtained by stimulation of the stratum radiatum with a 100-μs pulse of 1 mA in intentsity and delivered every 10 s.

### 2.2. Microdialysis procedure

The microdialysis probe was continuously perfused with an artificial cerebrospinal fluid (ACSF), composed of 132.8 mM NaCl, 6.7 mM urea, 3.7 mM glucose, 3.0 mM KCl, 2.0 mM $CaCl_2$, 0.7 mM $MgCl_2$ and 24.6 mM $NaHCO_3$ (the solution was adjusted at pH 7.4). Perfusion flow was controlled by a microinfusion pump (CMA/100, CMA/Microdialysis, Sweden) at a constant flow rate of 2.0 μl/min. Three consecutive samples were collected every 10 min to assess basal levels, at least, 120 min after probe insertion. Thereafter, perfusion with drugs via the probe was done. The changes in perfusion medium were done by a liquid switch (CMA/110, CMA/Microdialysis). The samples were frozen and stored at −80°C until analysis.

### 2.3. Experimental protocol

Five rats served as control and received 100 mM $K^+$ alone for 30 min (control group). Six rats received 0.05 mM sodium nitroprusside + 100 mM $K^+$ for 30 min (0.05 mM sodium nitropusside group). Five rats received 0.5 mM sodium nitropusside + 100 mM $K^+$ for 30 min (0.5 mM sodium nitroprusside group). Five rats received 0.5 mM sodium nitroprusside + 5 mM diethyldithiocarbamate + 100 mM $K^+$ for 30 min (diethyldithiocarbamate group). Six rats received 0.5 mM sodium nitroprusside + 1 mM dithiothreitol + 100 mM $K^+$ for 30 min (dithiothreitol group). Sodium nitroprusside, diethyldithiocarbamate or dithiothreitol was applied 30 min before perfusion with 100 mM $K^+$ for 30 min and thus the total perfusion time was 60 min. Dialysates were collected every 10 min.

### 2.4. Assay of aspartate and adenosine in the perfusate

Concentrations of aspartate in the perfusates were subsequently determined by reversed phase high performance liquid chromatography (HPLC) analysis using precolumn derivatization with *o*-phthaldialdehyde and a fluorescence detector (CMA/280, CMA/Microdialysis). A capillary column (C-18, BAS, Japan) was used. The mobile phase was 75% phosphate buffer (pH 6.0) with 0.1 mM EDTA–2Na, 10% acetonitrile and 3% tetrahydrofuran and was pumped at a flow rate of 60 μl/min. The amounts of aspartate were determined by measuring the peak area with reference to external standards.

Adenosine was analyzed using an HPLC with ultraviolet absorbance detector (UV-110, BAS) at 254 nm. A capillary column (C-18, BAS) was used. The mobile phase was 20 mM phosphate buffer (pH 5.5) containing 0.1 mM EDTA and 6% methanol and was pumped at a flow rate of 60 μl/min. Peak height was measured to determine the amount of adenosine.

### 2.5. In vitro recovery experiments

The percent recovery of aspartate and adenosine in vitro from the dialysis probe of 2 mm long membrane was evaluated by perfusing the dialysis probe with ACSF at 2.0 μl/min through a bath medium containing ACSF with aspartate or adenosine of 1.0 to 5.0 mM (maintained at 37.0°C). The constant relative recovery ratios for aspartate and adenosine were 12.4 ± 0.5% ($n = 5$) and 11.1 ± 0.3% ($n = 6$), respectively, with standard aspartate and adenosine.

### 2.6. Electrophysiological measurements

Extracellular field potentials were amplified using an AC amplifier (Nihon Kohden, AVB-10), visualized on a

memory oscilloscope (Nihon Kohden, VC-10). The hippocampal EEG activity was amplified using a DC amplifier (Nihon Kohden, MEZ-8201) and recorded on a polygraph (Nihon Kohden, V-85) and stored on a cassette data recorder (Nihon Kohden, RMG-5104). The population spikes were evoked by stimulation of Schaffer collateral–commissural fibers and recorded from the CA1 pyramidal cell layer with a glass-microelectrode placed nearby the dialysis probe.

### 2.7. Drugs

Sodium nitroprusside, diethyldithiocarbamate and dithiothreitol were analytical grade and purchased from Sigma (St. Louis, MO, USA). These compounds were dissolved in ACSF or ACSF containing high $K^+$ and then diluted to various concentrations.

### 2.8. Statistical analysis

The data are presented as means ± S.E.M. To determine the difference between experimental groups and time course of release of aspartate and adenosine, statistical analysis was performed by mixed type analysis of variance (ANOVA) with repeated measures followed by post hoc Bonferroni/Dunn's or Fisher's protected least significant difference (PLSD) tests, as indicated in the figure legends. A $P$ value $< 0.05$ was considered significant.

## 3. Results

### 3.1. Electrophysiological findings during development of sodium nitroprusside-induced seizure

Fig. 1 shows hippocampal EEG and population spikes under application of sodium nitroprusside. In the control group, spreading depression characterized by negative DC shifts repetitively occurred during 100 mM $K^+$ perfusion for 30 min (Fig. 1A). The population spikes disappeared during spreading depression, but recovered almost completely after high $K^+$ perfusion without occurrence of seizures. The coperfusion with 0.05 mM sodium nitroprusside and 100 mM $K^+$ induced only a small increase in population spike amplitude during washout of the drug without occurrence of seizures (Fig. 1B). The coperfusion with 0.5 mM sodium nitroprusside and 100 mM $K^+$ induced multiple population spikes and seizures, which were composed of high frequency, high amplitude spike discharges followed by burst discharges (sodium nitroprusside-induced seizures) during washout of sodium nitroprusside (Fig. 1C). Perfusion with 5 mM diethyldithiocarbamate, the NO trapper, completely abolished the sodium nitroprusside-induced seizures and perfusion with 1 mM dithiothreitol, the superoxide radical scavenger, reduced the frequency of the sodium nitroprusside-induced seizures

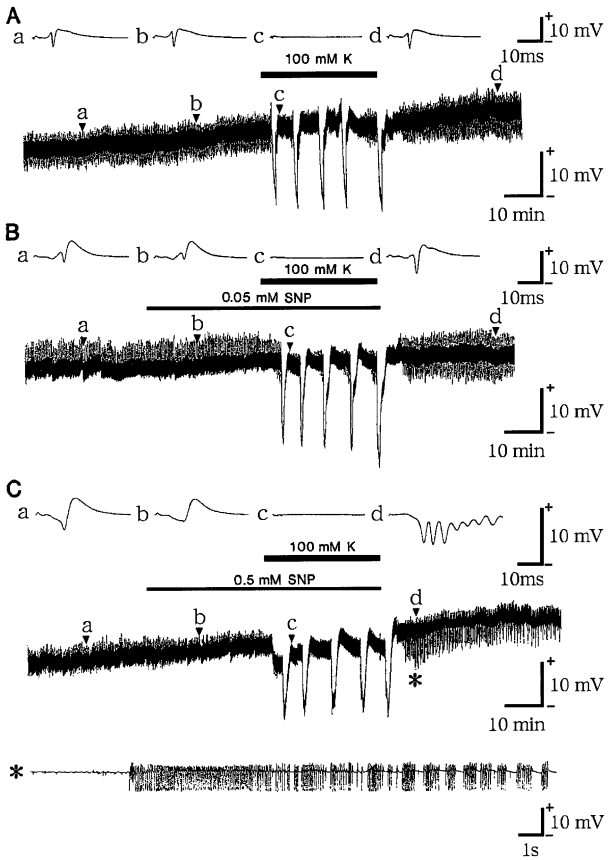


Fig. 1. Representative electrophysiological recordings from the hippocampal CA1 area, in which the seizures were induced by perfusion with sodium nitroprusside (sodium nitroprusside-induced seizures). In A, B and C, the first trace shows consecutive population spikes recorded at the time point, marked by lettered arrow heads, displayed here at a faster sweep. The second trace shows DC potential recordings on which the population spikes are superimposed. In C, the lowest trace shows the EEG record displayed at a faster sweep with the seizure discharges occurring at the time point marked by an asterisk. Upward deflection denotes positivity. In the control group (A), spreading depression repetitively occurred during 100 mM $K^+$ alone perfusion for 30 min. The population spikes disappeared during spreading depression and then recovered almost completely without occurrence of seizures. Coperfusion with 0.05 mM sodium nitroprusside and 100 mM $K^+$ induced a small increase in population spike amplitude without occurrence of seizures during washout of sodium nitroprusside (SNP) (B). Coperfusion with 0.5 mM sodium nitroprusside and 100 mM $K^+$ induced multiple population spikes and seizures during washout of sodium nitroprusside (SNP) (C).

(Hada et al., 2000). The mean number of spreading depressions occurring during high $K^+$ perfusion was not altered by perfusion with 0.05 or 0.5 mM sodium nitroprusside (data not shown).

### 3.2. Effects of NO-related compounds on release of aspartate

The mean basal concentration of aspartate in each 10-min sample was 186 ± 5 nmol/l ($n = 81$). The release of aspartate increased quickly to ninefold above the basal concentration and reached maximal levels during the first

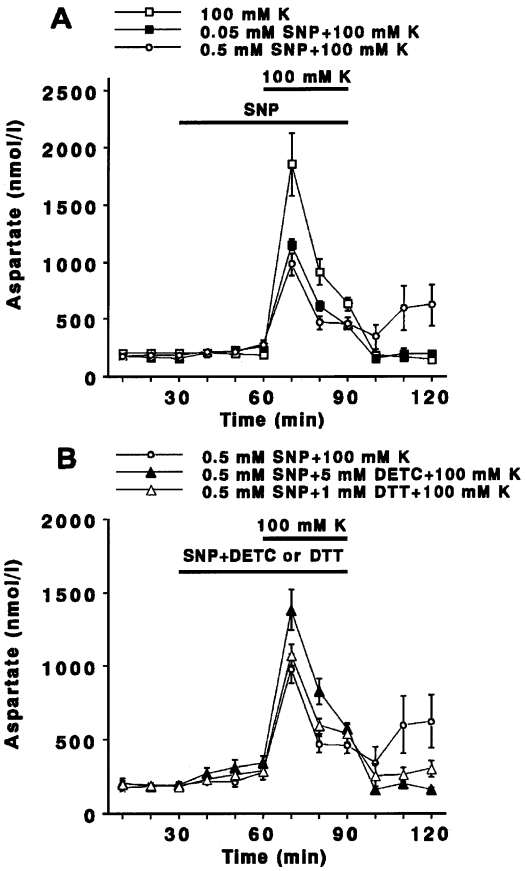


Fig. 2. (A) Time course for aspartate release evoked by perfusion with 100 mM $K^+$ alone (□; $n=5$), 0.05 mM sodium nitroprusside (SNP)+100 mM $K^+$ (■; $n=6$) or 0.5 mM sodium nitroprusside (SNP)+100 mM $K^+$ (○; $n=5$). Each point with a vertical bar represents the mean±S.E.M. for 5–6 experiments. ANOVA computed on aspartate release during high $K^+$ perfusion revealed that main effects of group and time and an interaction between group and time were significant ($F(2/13)=8.691$, $P=0.0040$; $F(2/26)=119.472$, $P<0.0001$; $F(4/26)=7.062$, $P<0.0005$, respectively). Group differences between control and 0.05 or 0.5 mM sodium nitroprusside were significant by post-hoc tests (Bonferroni/Dunn). ANOVA computed on aspartate release after high $K^+$ perfusion revealed that main effects of group and time and an interaction between group and time were significant ($F(2/13)=5.610$, $P=0.0175$; $F(2/26)=3.751$, $P=0.0370$; $F(4/26)=3.409$, $P=0.0228$, respectively). Group differences between 0.5 mM sodium nitroprusside and control or 0.05 mM sodium nitroprusside were significant by post-hoc tests (Bonferroni/Dunn). (B) Time course for aspartate release evoked by perfusion with 0.5 mM sodium nitroprusside (SNP)+100 mM $K^+$ (○; $n=5$) (the same group as that in A), 0.5 mM sodium nitroprusside (SNP)+5 mM diethyldithiocarbamate (DETC)+100 mM $K^+$ (▲; $n=5$) or 0.5 mM sodium nitroprusside (SNP)+1 mM diethiothreitol (DTT)+100 mM $K^+$ (△; $n=6$). ANOVA computed on aspartate release during high $K^+$ perfusion revealed that a main effect of group and an interaction between group and time were significant ($F(2/13)=4.920$, $P=0.0256$; $F(2/26)=118.425$, $P<0.0001$, respectively). A group difference between sodium nitroprusside and diethyldithiocarbamate was significant by a post-hoc test (Bonferroni/Dunn). ANOVA computed on aspartate release after high $K^+$ perfusion revealed that main effects of group and time were significant ($F(2/13)=3.849$, $P=0.0486$; $F(2/26)=3.837$, $P=0.0347$, respectively). A group difference between sodium nitroprusside and diethyldithiocarbamate was significant by a post-hoc test (Fisher's PLSD).

10 min of high $K^+$ perfusion (Fig. 2A). Compared with the control group, perfusion with 0.05 or 0.5 mM sodium nitroprusside significantly attenuated aspartate release during high $K^+$ perfusion. An increase in the aspartate release was observed during washout of 0.5 mM sodium nitroprusside. The increase in aspartate release related to the occurrence of seizures during washout of sodium nitroprusside

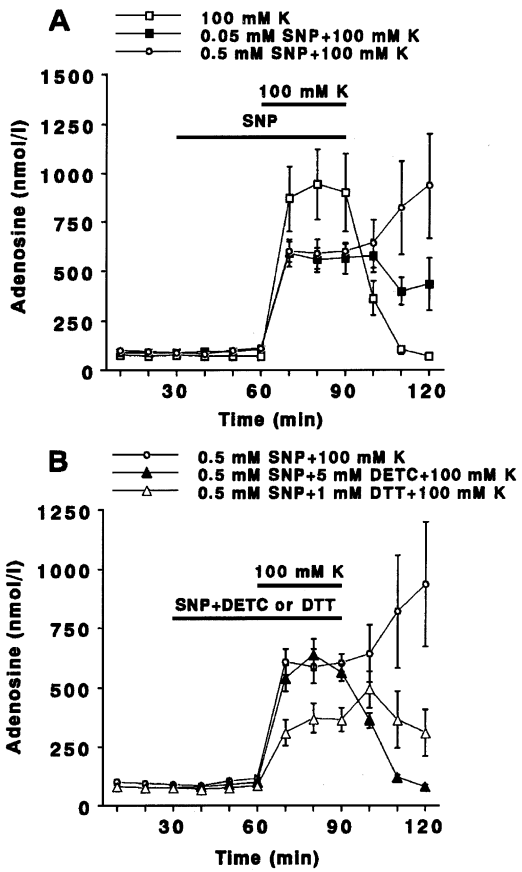


Fig. 3. (A) Time course for adenosine release evoked by perfusion with 100 mM $K^+$ alone (□; $n=5$), 0.05 mM nitroprusside (SNP)+100 mM $K^+$ (■; $n=6$) or 0.5 mM sodium nitroprusside (SNP)+100 mM $K^+$ (○; $n=5$). Each point with a vertical bar represents the mean±S.E.M. for 5–6 experiments. ANOVA computed on adenosine release after high $K^+$ perfusion revealed that a main effect of group and an interaction between group and time were significant ($F(2/13)=6.187$, $P=0.0129$; $F(4/26)=3.930$, $P=0.0126$, respectively). A group difference between control and 0.5 mM sodium nitroprusside was significant by a post-hoc test (Bonferroni/Dunn). (B) Time course for adenosine release evoked by perfusion with 0.5 mM sodium nitroprusside (SNP)+100 mM $K^+$ (○; $n=5$) (the same group as that in A), 0.5 mM sodium nitroprusside (SNP)+5 mM diethyldithiocarbamate (DETC)+100 mM $K^+$ (▲; $n=5$) or 0.5 mM sodium nitroprusside (SNP)+1 mM dithiothreitol (DTT)+100 mM $K^+$ (△; $n=6$). ANOVA computed on adenosine release during high $K^+$ perfusion revealed that a main effect of group was significant ($F(2/13)=8.492$, $P=0.0044$). Group differences between dithiothreitol and sodium nitroprusside or diethyldithiocarbamate were significant by post-hoc tests (Bonferroni/Dunn). ANOVA computed on adenosine release after high $K^+$ perfusion revealed that a main effect of group and an interaction between group and time were significant ($F(2/13)=5.861$, $P=0.0153$; $F(4/26)=6.545$, $P=0.0009$, respectively). Group differences between sodium nitroprusside and diethyldithiocarbamate or dithiothreitol were significant by post-hoc tests (Fisher's PLSD).

was reversed by 5 mM diethyldithiocarbamate in the presence of 0.5 mM sodium nitroprusside (Fig. 2B).

### 3.3. Effects of NO-related compounds on release of adenosine

The mean basal concentration of adenosine in each 10 min sample was $82 \pm 3$ nmol/l ($n = 81$). Release of adenosine increased up to 13-fold during the second 10 min of high $K^+$ perfusion (Fig. 3A). The release of adenosine returned to the basal level within 20 min after the onset of reperfusion with ACSF. Compared with the control group, perfusion with 0.05 mM sodium nitroprusside significantly attenuated adenosine release during high $K^+$ perfusion. Adenosine release was significantly enhanced during washout of 0.5 mM sodium nitroprusside, compared with the control group. The enhancement of adenosine release related to the occurrence of seizures was almost completely reversed by 5 mM diethyldithiocarbamate and partially by 1 mM dithiothreitol (Fig. 3B).

## 4. Discussion

The present study has shown for the first time that seizures induced by intrahippocampal perfusion with 0.5 mM sodium nitroprusside (NO donor) are closely associated with increases in release of aspartate and adenosine during washout of sodium nitroprusside and that the effects of sodium nitroprusside are reversed by diethyldithiocarbamate (NO trapper). In addition, we have observed that NO induces adenosine release in the early stages of neuronal excitation.

The finding that intrahippocampal perfusion with sodium nitroprusside induces seizures is in accordance with other reports is showing that intrahippocampal microinjection of sodium nitroprusside produced epileptogenic discharges in freely moving rats (Bagetta et al., 1993) and that microinjection of sodium nitroprusside into the rat deep prepiriform cortex induced seizures (De Sarro et al., 1993). On the contrary, Marangoz et al. (1994) reported that intracortical microinjection of sodium nitroprusside decreased epileptiform discharges elicited by penicillin. In our previous study, 0.5 mM sodium nitroprusside, but not 0.05 mM, always induced seizures, which were abolished completely by diethyldithiocarbamate and partially by dithiothreitol (superoxide radical scavenger) (Hada et al., 2000). Therefore, we think that both NO itself and, in part, peroxynitrite are responsible for generation of the sodium nitroprusside-induced seizures.

The present study has also shown that perfusion with 0.05 mM sodium nitroprusside reduces high $K^+$-evoked adenosine release from rat hippocampus. This result has been related to other reports showing that a large component of high $K^+$-evoked adenosine release seems to be caused by activation of NMDA receptors following the release of excitatory amino acids (Chen et al., 1992; Pazzagli et al., 1994) and that NO has been shown to inhibit NMDA receptor-mediated physiological responses (Hoyt et al., 1992). In the present study, we have also shown that sodium nitroprusside attenuates high $K^+$-evoked release of aspartate. This finding is also in accordance with other reports showing that sodium nitroprusside dose-dependently inhibits the depolarization-evoked glutamate release (Kamisaki et al., 1995; Sequeira et al., 1999).

The increase in the extracellular adenosine was closely associated with the development of seizures in rat hippocampus during washout of sodium nitroprusside. During and Spencer (1992) observed the 6- to 31-fold rise in hippocampal adenosine level during complex partial seizures in humans. Berman et al. (2000) have recently shown that different seizure models are associated with increased dorsal hippocampal adenosine release in rats. Fallahi et al. (1996) have shown that an NO donor, *S*-nitroso-*N*-acetylpenicillamine, can remarkably enhance electrically evoked release of endogenous adenosine. Rosenberg et al. (2000) have provided in rat forebrain neurons in culture that NO itself may stimulate an increase in extracellular adenosine accumulation. The rate of adenosine formation has also risen during enhancement of neuronal activity (Jonzon and Fredholm, 1985; Mitchell et al., 1993). It has been proposed that sodium nitroprusside significantly increases the activity of neuronal persistent $Na^+$ channels in cultured hippocampal neurons (Hammarstrom and Gage, 1999). The increase in persistent $Na^+$ current would cause membrane depolarization and increase the firing rate of action potentials. Thus, sodium nitroprusside may enhance the release of adenosine, which is related to the increased neuronal activity. The increase in extracellular adenosine would be probably caused by enhancement of adenosine release, but not by inhibition of adenosine metabolism, because the extracellular inosine as a metabolite of adenosine similarly increased during washout of sodium nitroprusside (our unpublished observations). Furthermore, adenosine release is likely to be triggered by an increase in both NO itself and in part peroxynitrite, which results in reaction with superoxide radicals, because sodium nitroprusside-induced adenosine release was almost completely reversed by the NO trapper, diethyldithiocarbamate, and partially by the superoxide radical scavenger, dithiothreitol, in the present study. These findings also are in line with the following reports. It has been shown that NO can evoke basal adenosine release from in vivo rat striatum (Fischer et al., 1995) and rat hippocampal slices (Fallahi et al., 1996). In addition, it has recently been demonstrated that the production of free radicals is involved in the rise of adenosine release induced by kainate (Carswell et al., 1997) and NMDA (Delaney et al., 1998). It is not clear how NO evokes adenosine release. However, excessive NO has previously been shown to cause glutamate release following mitochondrial dysfunction leading to an energy deficiency state (McNaught and Brown,

1998). Under these conditions, ATP catabolism would be facilitated and consequently adenosine levels would be enhanced.

Many physiological actions of NO in the nervous system have been mediated by stimulation of guanylyl cyclase (Garthwaite, 1991). Recently, sodium nitroprusside has been shown to enhance cyclic GMP formation in the rat hippocampal synaptosomes (Sequeira et al., 1999). Because cyclic GMP has been found to increase the firing rate of neurons (Stone and Taylor, 1977), the enhanced cyclic GMP formation may be involved in the sodium nitroprusside-induced adenosine release. Furthermore, it has been demonstrated that the NO donor, *S*-nitroso-*N*-acetylpenicillamine, inhibits synaptic transmission in the rat hippocampus through an NO-cyclic GMP signalling pathway and an adenosine receptor-dependent mechanism (Broome et al., 1994). Thus, NO-induced adenosine release may be related to enhancement of cyclic GMP formation.

We have demonstrated in the present study that the increase in the extracellular adenosine release is closely related to the development of sodium nitroprusside-induced seizures in rat hippocampus. Adenosine is a neuromodulator that presynaptically blocks the release of glutamate (Burke and Nadler, 1988) and postsynaptically hyperpolarizes pyramidal cells in the hippocampal CA1 region via adenosine $A_1$ receptors (Siggins and Schubert, 1981). Thus, NO-induced endogenous adenosine probably has a neuroprotective action under pathological conditions such as epilepsy. The idea is supported by other reports showing that adenosine has protective effects against epilepsy (Dunwiddie, 1999).

In conclusion, the present study shows that, in rat hippocampal CA1 pyramidal neurons, an increase in adenosine release is closely related to development of sodium nitroprusside-induced seizures, which are triggered by an increase in both NO itself and in part peroxynitrite, which results in reaction with superoxide radicals.

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