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Role of peroxynitrite in [^{3}H]γ-aminobutyric acid release evoked by nitric oxide and its mechanism

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Abstract

Role of peroxynitrite in [^{3}H]γ-aminobutyric acid (GABA) release evoked by *N*-methyl-D-aspartate (NMDA) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) and mechanisms of [^{3}H]GABA release induced by peroxynitrite in comparison with those induced by NMDA and SNAP were investigated using cerebrocortical neurons. NMDA dose dependently increased [^{3}H]GABA release, which was significantly inhibited by hemoglobin and superoxide scavengers, Cu^{2+}, Zn^{2+}-superoxide dismutase and ceruloplasmin. The NMDA-evoked [^{3}H]GABA release was significantly suppressed by GABA transport inhibitors and inhibitors of voltage-dependent L-typed Ca^{2+} channel. The SNAP-evoked [^{3}H]GABA release was significantly reduced by Ca^{2+} withdrawal and by GABA transport inhibitors either in the presence or absence of Ca^{2+}. Similar patterns of [^{3}H]GABA release induced by peroxynitrite were observed. These results indicate that peroxynitrite formed by the reaction of NO with superoxide participates, in part, in the release of [^{3}H]GABA induced by NMDA and SNAP.

Keywords: Nitric oxide (NO); Peroxynitrite; GABA (γ-aminobutyric acid) release; Superoxide; Cerebral cortical neuron

1. Introduction

Nitric oxide (NO), an unstable radical, plays a variety of roles in mammalian cells. NO serves as a mediator in non-adrenergic and non-cholinergic (NANC) neurotransmission (Rand, 1992; Sanders and Ward, 1992) in the peripheral nervous system and tissues, functions as an effector molecule in immunological reactions mediated by macrophages and neutrophils, and inhibits platelet coagulation and platelet adhesion to collagen fibrils and endothelial cell matrix (Moncada et al., 1991). NO has been also reported to have several functions in the central nervous system (CNS). NO is formed via NADPH-dependent oxidative deamination of L-arginine, which is catalyzed by NO synthase (Knowels et al., 1989; Bredt and Snyder, 1990; Bredt et al., 1991, Nathan, 1992). NO formed in neurons is released and acts on neighbouring cells (Garthwaite, 1991). In addition, recent lines of evidence indicate the possible involvement of NO in long-term potentiation in the hippocampus, in neuronal injury induced by glutamate and *N*-methyl-D-aspartate (NMDA), and in release of neurotransmitters (Garthwaite, 1991; Snyder, 1992; Lonart et al., 1992; Prast and Philippu, 1992; Zhu and Luo, 1992).

NO reacts rapidly with superoxide to form peroxynitrite at a rate of at least 6.7×10^9 $M^{-1} \cdot s^{-1}$ (Blough and Zafiriou, 1985; Huie and Padmaja, 1993). Peroxynitrite possesses a more potent oxidizing activity than superoxide, and participates in alterations in cell functions subsequent to the oxidation of cellular components (Beckman et al., 1990; Radi et al., 1991a; Hogg et al., 1992; Ischiropoulos et al., 1992a). These data suggest a possibility that NO produced in neurons reacts with superoxide to form peroxynitrite and this formed peroxynitrite, in turn, induces the release of neurotransmitter. In this study, we have, therefore, investigated the role of peroxynitrite in the release of γ-aminobutyric acid (GABA) evoked by NMDA and *S*-nitroso-*N*-acetylpenicillamine (SNAP) and the mechanisms of peroxynitrite-evoked [^{3}H]GABA release in comparison with NMDA- and SNAP-induced [^{3}H]GABA releases.

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2. Materials and methods

2.1. Primary culture of cerebral cortical neurons

The procedures to isolate and primary culture of cerebral cortical neurons were previously described (Ohkuma et al., 1986) with a minor modification. In brief, the neopallium free of meninges was dissected from 15-day-old fetuses of ddY strain mice, minced, trypsinized and centrifuged. Cells thus obtained were suspended in Dulbecco's modified Eagle medium (DMEM: pH 7.4) supplemented with 15% fetal calf serum to adjust the cell number to 3.0×10^6 cells/ml of cell suspension, and 1 ml of the cell suspension was added into a culture dish (Falcon 'Primaria': 35 mm in diameter). Prior to the addition of the cell suspension, a culture dish was treated with poly-L-lysine (molecular weight; approximately 70 kDa, 5 μg/ml of distilled water) at 37°C for 12 h and then rinsed twice with Hank's solution (pH 7.4; 137 mM NaCl, 5.4 mM KCl, 1.26 mM $CaCl_2$, 0.64 mM $Na_2HPO_4 \cdot 12H_2O$, 0.44 mM KH_2PO_4, 0.62 mM $MgSO_4 \cdot 6H_2O$, 3.0 mM $NaHCO_3$, 5.5 mM glucose). The cells were thereafter incubated at 37°C under humidified 95% air-5% CO_2 for 3 days followed by the incubation of cells with 10 μM cytosine arabinoside in DMEM containing 10% horse serum for 1 day. Thereafter, the neurons were incubated in DMEM supplemented with 10% horse serum under the same conditions described above. The culture medium was exchanged every 4 days. The proportion of the neurons among the cells thus obtained was more than 95% (Ohkuma et al., 1986; Kuriyama et al., 1987). The neurons of 13-day-old in culture were used in each experiment. The neurons used here were reported to have NMDA receptors (Ohkuma et al., 1994a). About 80% of the neurons were confirmed to be GABAergic neurons, when examined immunohistochemically with anti-glutamate dehydrogenase (GAD) antibody (Kuriyama et al., 1987).

2.2. Measurement of [^{3}H]GABA release from neurons

The protocol for examining the release of [^{3}H]GABA is described as follows. The neurons were incubated with 2 nM [^{3}H]GABA (containing 1.0 μCi [^{3}H]GABA/dish) in Krebs-Ringer bicarbonate buffer (KRB: pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 2.7 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $MgSO_4 \cdot 6H_2O$, 10 mM glucose and 25 mM $NaHCO_3$) containing 20 mM Hepes (Hepes-KRB: pH 7.4) at 37°C for 30 min. After this preincubation, the neurons were rinsed three times with ice-cold Hepes-KRB and further incubated in Hepes-KRB at 37°C for 10 min. Thereafter, the neurons were incubated with fresh and warm (37°C) Hepes-KRB at an interval of 5 min for a total of 25 or 50 min. When examining the effect of NMDA on [^{3}H]GABA release, Mg^{2+}-free Hepes-KRB was used as the incubation buffer to minimize the inhibitory effect of Mg^{2+} on the function of NMDA receptors. Both Hepes-KRB and Mg^{2+}-free Hepes-KRB were supplemented with 100 μM aminooxyacetic acid to prevent the degradation of [^{3}H]GABA incorporated into the neurons and released into the incubation buffer. An aliquot of the sample collected during each interval was used to measure the radioactivity released from the neurons into the incubation buffer by liquid scintillation spectrometry. The agents such as NMDA, SNAP and peroxynitrite were added at the beginning of the 4th interval of the incubation. Cu^{2+}, Zn^{2+}-superoxide dismutase (SOD), ceruloplasmin, hemoglobin, (±)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*d*]cyclohepten-5,10-imine (MK-801), GABA transport inhibitors such as nipecotic acid and 1-(2-(((diphenylmethylene)amino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid (NO-711) (Dong et al., 1984) were added into the incubation buffer 15 s before the addition of NMDA and SNAP. The release of [^{3}H]GABA during the 3rd interval was defined as the basal release, and stimulated release was expressed as a percentage of the basal release. The basal release obtained by the use of Hepes-KRB was not significantly different from that by the use of Mg^{2+}-free Hepes-KRB.

When examining the time course of [^{3}H]GABA releases evoked by NMDA, SNAP and peroxynitrite, an aliquot of the incubation buffer collected every 1 min (1-min fraction) during the 3rd and 4th intervals was used to measure the radioactivity released into the incubation buffer as described above.

To evaluate toxic effects of NMDA and peroxynitrite on neuronal function, we have checked whether the ability of the neurons to release [^{3}H]GABA during the 1st stimulation differs from that during the 2nd stimulation. Each agent was added immediately after the initiation of the 4th and 9th intervals of the incubation. The radioactivities of [^{3}H]GABA released during the 4th and 9th intervals were defined as s_1 and s_2 respectively, and those during the 3rd and 8th intervals were defined as the basal releases (b_1 and b_2) against s_1 and s_2, respectively. The ratios of s_1 to b_1 and s_2 to b_2 were defined as S_1 and S_2, respectively. In addition, the leakage of lactic dehydrogenase (LDH) activity from the neurons and the activity of the neurons to exclude trypan blue dye were examined after the exposure of the neurons to 100 μM NMDA, 5 μM SNAP and 10 μM peroxynitrite according to the previously reported methods (Ohkuma et al., 1994b).

2.3. Synthesis of peroxynitrite

Peroxynitrite was synthesized according to the method of Beckman et al. (Beckman et al., 1990). The resultant alkaline solution obtained contained about 100 mM peroxynitrite, which was determined by an absorbance at 302 nm ($\epsilon_{302} = 1670\ M^{-1} \cdot cm^{-1}$) (Hughes and Nicklin, 1968). Peroxynitrite was used immediately after the synthesis. The starting materials to synthesize peroxynitrite, hydrogen peroxide and sodium nitrite, and decomposed sub-

stances of peroxynitrite showed no effects on [^{3}H]GABA release (Ohkuma et al., 1995b).

2.4. Protein measurement

After the release experiments, the neurons were scraped off with 0.5 N NaOH, and the content of protein in the alkaline-digested neurons was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

2.5. Statistical analysis

The data were expressed as the means ± S.E.M. and statistical significance was analyzed as described in each legend of the figures and tables following the application of the one-way analysis of variance (ANOVA).

2.6. Materials

[^{3}H]GABA (185 MBq/mmol) was purchased from New England Nuclear (Boston, USA). NMDA, MK-801, SNAP and NO-711 were obtained from Research Biochemical (Natick, USA). Hazelton Research Products (Lenexa, USA) was the source of fetal calf and horse serums. SOD, ceruloplasmin and hemoglobin were obtained from Sigma Chemicals (St. Louis, USA). Other chemicals used were locally available and of analytical grade.

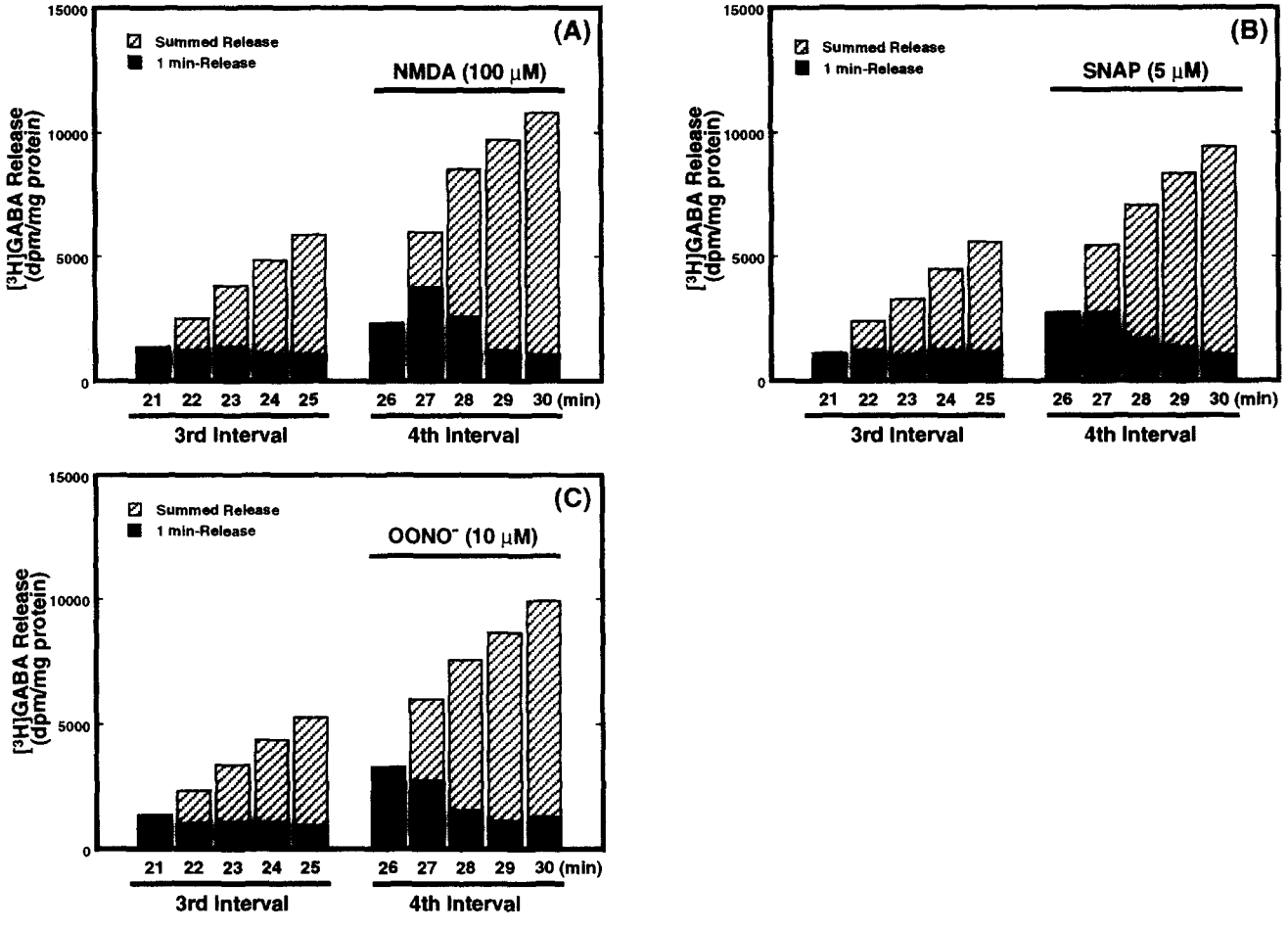

Fig. 1. Time course of (A) *N*-methyl-D-aspartate (NMDA)-, (B) *S*-nitroso-*N*-acetylpenicillamine (SNAP)- and (C) peroxynitrite ($OONO^-$)-evoked [^{3}H]GABA releases from cerebral cortical neurons. After the neurons were preincubated with 2 nM [^{3}H]GABA (1.0 μCi [^{3}H]GABA/dish) in Krebs-Ringer bicarbonate buffer containing 20 mM Hepes (Hepes-KRB, pH 7.4) at 37°C for 30 min followed by washing the cells three times with ice-cold Hepes-KRB, the neurons were preincubated in Hepes-KRB at 37°C for 10 min. Thereafter, the neurons were incubated in Hepes-KRB at 37°C at an interval of 5 min for a total of 25 min. When examining the effect of NMDA, Mg^{2+}-free Hepes-KRB was used as the incubation buffer to prevent the inhibitory actions of Mg^{2+} on functions of NMDA receptors. To investigate the effect of the agents, the agents were usually added at the initiation of the 4th interval of the incubation. After the addition of each agent into the incubation buffer, an aliquot of the incubation buffer was collected every 1 min (1-min release) and was used to measure the radioactivity released into the incubation buffer. Similar procedures were employed to measure 1-min release during the 3rd interval. Each value represents the mean of 4 separate experiments, each of which was carried out in triplicate and the value of the S.E.M was less than 8% of each mean value.

3. Results

3.1. Time course of [^{3}H]GABA releases evoked by NMDA, SNAP and peroxynitrite

As shown in Fig. 1, the releases of [^{3}H]GABA stimulated by NMDA, SNAP and peroxynitrite reached their plateaus within 1 or 2 min after the addition of these agents into the incubation buffer, and 5 min after the addition of the agents the 1-min releases decreased to the levels similar to that determined in the absence of the agents. The ratios of radioactivity determined in 1 min release of the basal level to total radioactivity (remained radioactivity in the neurons plus radioactivity released into the incubation buffer) in all cases were about 0.15% (data not shown). Based on these data, we have employed 5 min as the duration to stimulate [^{3}H]GABA release in this study.

3.2. Pharmacological characteristics of NMDA-induced [^{3}H]GABA release

NMDA produces NO by activating NO synthase via its receptor stimulation in the CNS (Bredt and Snyder, 1989; Kiedrowski et al., 1992). As shown in Fig. 2A, NMDA increased the release of [^{3}H]GABA from the cerebral cortical neurons in a dose-dependent manner and the maximal release was observed at 100 μM of NMDA. MK-801,

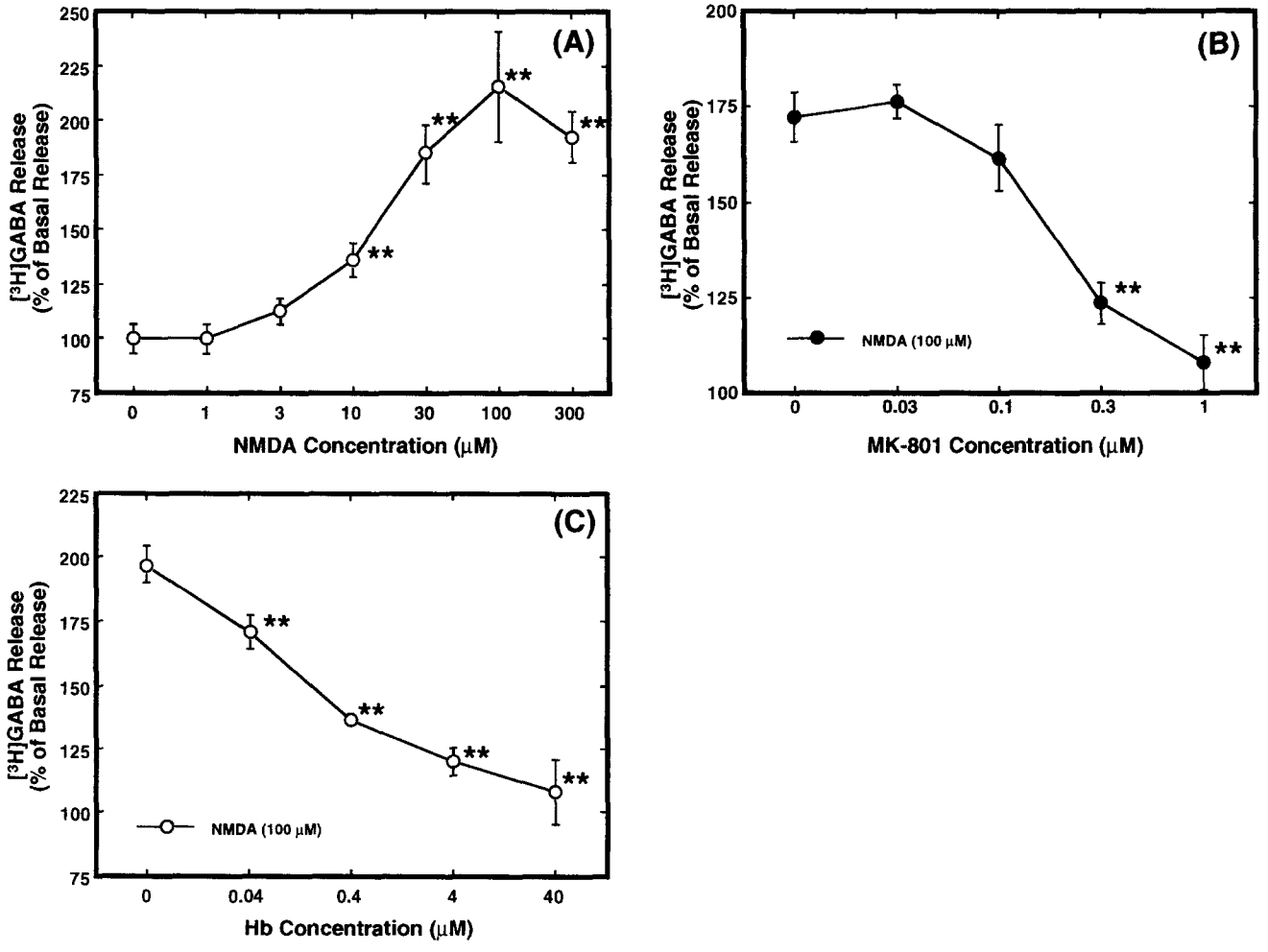

Fig. 2. Characteristics of *N*-methyl-D-aspartate (NMDA)-evoked [^{3}H]GABA releases from cerebral cortical neurons. The procedures to measure the release of [^{3}H]GABA are described in Materials and methods in detail. Each value represents the mean ± S.E.M. obtained from 5 separate experiments, each of which was carried out in triplicate and is expressed as a percentage of the basal release (the release during the 3rd interval). (A) Effect of NMDA on [^{3}H]GABA release. The basal release was 4,263 ± 643 dpm/mg protein/interval. ** $P < 0.01$, compared with the basal release (Dunnett's test). (B) Effect of (±)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*d*]cyclohepten-5,10-imine (MK-801) on NMDA (100 μM)-induced release of [^{3}H]GABA from cerebral cortical neurons. MK-801 was added into the incubation buffer immediately before the addition of NMDA. The basal release was 5109 ± 454 dpm/mg protein/interval. ** $P < 0.01$, compared with the release determined in the presence of NMDA alone (Dunnett's test). (C) Effect of hemoglobin (Hb) on NMDA-induced release of [^{3}H]GABA from cerebral cortical neurons. Hemoglobin was added into the incubation buffer immediately before the addition of NMDA. The basal release was 4916 ± 563 dpm/mg protein/interval. * $P < 0.05$, ** $P < 0.01$, compared with the release determined in the presence of NMDA alone (Dunnett's test).

a non-competitive antagonist specific to the NMDA receptor complex, dose dependently inhibited the NMDA-induced [^{3}H]GABA release (Fig. 2B). Although NO synthase inhibitors such as N^{ω}-nitro-L-arginine and N^{G}-methyl-L-arginine at 100 μM showed no alterations in the basal release of [^{3}H]GABA, they significantly reduced the NMDA-induced [^{3}H]GABA release (Table 1), indicating that the increase in [^{3}H]GABA release induced by NMDA is mediated by the NO production via the NMDA receptor activation. Fig. 2C shows the effect of hemoglobin on the NMDA-induced [^{3}H]GABA release. Hemoglobin reduced the NMDA-induced [^{3}H]GABA release in a dose-dependent manner. The NMDA-evoked [^{3}H]GABA release was inhibited by almost 100% in the presence of 40 μM hemoglobin (Fig. 2C).

The removal of Ca^{2+} from the incubation buffer abolished completely the stimulatory action of NMDA on [^{3}H]GABA release (Fig. 3A). In addition, nipecotic acid and NO-711 decreased significantly the NMDA-evoked release in a dose-dependent manner, and the inhibitions by nipecotic acid and NO-711 on the NMDA-evoked [^{3}H]GABA release reached their plateaus at concentrations of 1 and 0.1 μM, respectively (Fig. 3A). Interestingly, voltage-dependent L-typed Ca^{2+} channel inhibitors, verapamil and nifedipine, with nipecotic acid or NO-711 reduced the NMDA-induced [^{3}H]GABA release to the level of the basal release (Fig. 3A).

3.3. Pharmacological characteristics of SNAP-induced [^{3}H]GABA release

We have already demonstrated that SNAP has a capacity to evoke [^{3}H]GABA release and that this stimulatory action is abolished by hemoglobin (Ohkuma et al., 1995b).

The effects of the removal of Ca^{2+} from the incubation buffer and of GABA transport inhibitors on the SNAP-induced [^{3}H]GABA release were examined. As shown in Fig. 3B, the removal of Ca^{2+} reduced the [^{3}H]GABA release evoked by SNAP by about 50%. In addition, both nipecotic acid and NO-711 significantly inhibited the SNAP-evoked [^{3}H]GABA release in either the presence or absence of Ca^{2+} (Fig. 3B).

Table 1
Effects of NO synthase inhibitors on NMDA-induced release of [^{3}H]GABA from cerebral cortical neurons

Agent (100 μM)	[^{3}H]GABA release (% of basal release)	
	−NMDA (100 μM)	+NMDA (100 μM)
None	100.0 ± 5.7	196.4 ± 3.0 [a]
Nitro-arginine	104.8 ± 12.9	99.8 ± 12.1 [b]
Methyl-arginine	102.1 ± 10.1	104.0 ± 7.0 [b]

The procedures to measure the release of [^{3}H]GABA are described in Materials and methods in detail. NO synthase inhibitors were present in the incubation buffer during the last 25 min of the incubation. The basal release of [^{3}H]GABA was 5552 ± 316 dpm/mg protein/interval. Each value represents the mean ± S.E.M. obtained from 5 separate experiments, each of which was carried out in triplicate and is expressed as a percentage of the basal release. [a] $P < 0.01$, compared with the value determined in the presence of NMDA alone (Scheffé's test). [b] $P < 0.01$, compared with the basal release (Scheffé's test). Nitro-arginine: N^{ω}-nitro-L-arginine, Methyl-arginine: N^{G}-methyl-L-arginine.

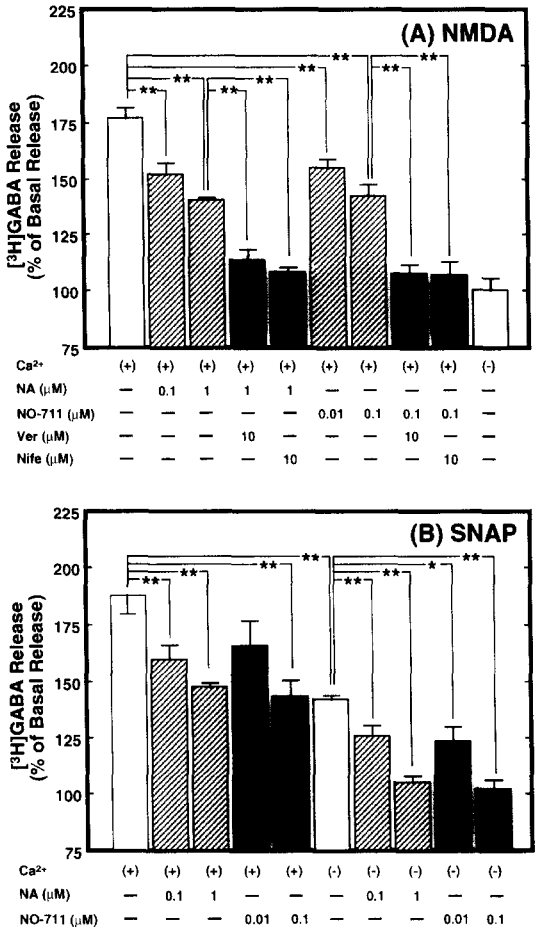

Fig. 3. Effects of Ca^{2+} withdrawal, GABA transport inhibitors and inhibitors of voltage-dependent L-typed Ca^{2+} channel on (A) *N*-methyl-D-aspartate (NMDA: 100 μM)- and (B) *S*-nitroso-*N*-acetylpenicillamine (SNAP: 5 μM)-induced release of [^{3}H]GABA from cerebral cortical neurons. The procedures to measure the release of [^{3}H]GABA are described in Materials and methods in detail. Each value represents the mean ± S.E.M. obtained from 4 separate experiments, each of which was carried out in triplicate and is expressed as a percentage of the basal release (the release during the 3rd interval). The basal releases for (A) and (B) were 6121 ± 320 and 5791 ± 297 dpm/mg protein/interval, respectively. * * $P < 0.01$ (Bonferroni's test). NA; nipecotic acid, Ver; verapamil, Nif; nifedipine.

3.4. Effects of Cu^{2+}, Zn^{2+}-superoxide dismutase (SOD) and ceruloplasmin on NMDA-induced [^{3}H]GABA release

SOD inhibited dose dependently the NMDA-induced [^{3}H]GABA release (Fig. 4A). The release evoked by NMDA was completely abolished in the presence of SOD at a concentration of 10 units/ml. On the other hand, SOD (10 units/ml) alone showed no effects on the basal release of [^{3}H]GABA (data not shown). Similarly, the release of

[^{3}H]GABA induced by NMDA was reduced by ceruloplasmin, whereas ceruloplasmin alone did not show any alterations in the basal release of [^{3}H]GABA (Fig. 4B).

Similar inhibitory actions of superoxide scavengers on the SNAP-induced [^{3}H]GABA release were also reported (Ohkuma et al., 1995b).

3.5. Pharmacological characteristics of synthesized peroxynitrite on [^{3}H]GABA release from cerebral cortical neurons

The data that synthesized peroxynitrite increases dose dependently [^{3}H]GABA release from the cerebral cortical neurons have been demonstrated (Ohkuma et al., 1995b).

The stimulatory effect of peroxynitrite on [^{3}H]GABA release in the absence of Ca^{2+} was reduced by about 50%, when compared with that observed in the presence of Ca^{2+} (Fig. 5). Nipecotic acid and NO-711 inhibited dose dependently the peroxynitrite-induced [^{3}H]GABA release either in the presence or absence of Ca^{2+} (Fig. 5). In addition, a simultaneous treatment of the neurons with Ca^{2+} removal and the addition of nipecotic acid or NO-711 reduced the peroxynitrite-induced [^{3}H]GABA release to the level of the basal release (Fig. 5).

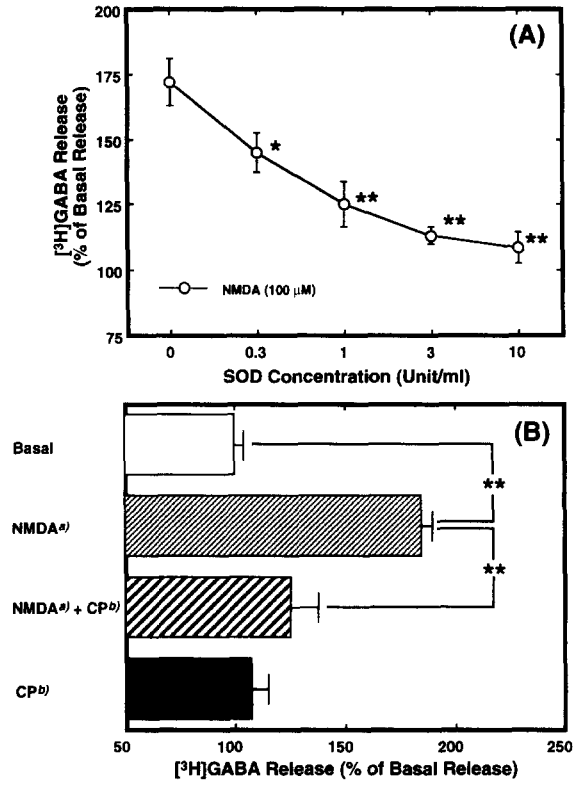

Fig. 4. Effects of (A) Cu^{2+}, Zn^{2+} superoxide dismutase (SOD) and (B) ceruloplasmin (CP) on *N*-methyl-D-aspartate (NMDA: 100 μM)-induced release of [^{3}H]GABA from cerebral cortical neurons. The procedures to measure the release of [^{3}H]GABA are described in Materials and methods in detail. SOD and CP were added into the incubation buffer immediately before the addition of NMDA. Each value represents the mean ± S.E.M. obtained from 5 separate experiments, each of which was carried out in triplicate and is expressed as a percentage of the basal release (the release during the 3rd interval). The basal releases for (A) and (B) were 4786 ± 607 and 4293 ± 521 dpm/mg protein/interval, respectively. (A) * $P < 0.05$, ** $P < 0.01$, compared with the release determined in the presence of NMDA alone (Dunnett's test). (B) ** $P < 0.01$ (Scheffé's test). [a] 100 μM, [b] 5 μM.

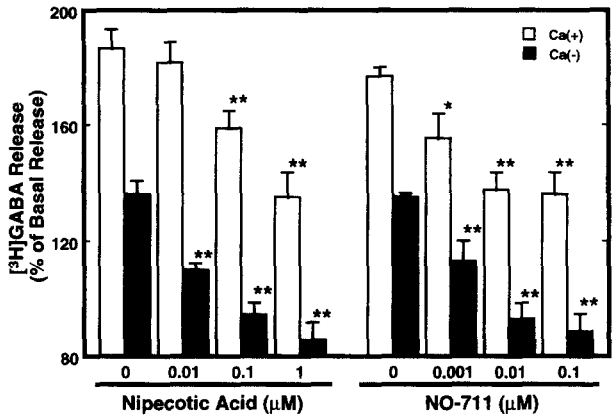

Fig. 5. Effects of Ca^{2+} withdrawal and GABA transport inhibitors on peroxynitrite (10 μM)-evoked [^{3}H]GABA release. The procedures to measure the release of [^{3}H]GABA are described in Materials and methods in detail. Each value represents the mean ± S.E.M. obtained from 4 separate experiments, each of which was carried out in triplicate and is expressed as a percentage of the basal release (the relse during the 3rd interval). The basal release was 6231 ± 336 dpm/mg protein/interval. * $P < 0.05$, ** $P < 0.01$, compared with each value determined in the presence of peroxynitrite alone (Dunnett's test).

3.6. Evaluation for deterioration of the process of GABA release from cerebral cortical neurons by NMDA and peroxynitrite during release experiments

Because agents such as NMDA and peroxynitrite are considered to show neurotoxicity, we have checked the effects of these agents on the neuronal function by examining the releases of [^{3}H]GABA after two times stimulations of the neurons with each agent, and the difference between the [^{3}H]GABA releases upon the 1st stimulation and that upon the 2nd stimulation was compared. As shown in Table 2, the ratios of S_2 evoked by NMDA and peroxynitrite to the respective S_1 were almost 1.0. In addition, the releases of [^{3}H]GABA during the intervals between the 1st and the 2nd stimulations were similar and were relatively smaller than the release detected during the 3rd interval (data not shown).

The ratios of the leaked LDH activity from the neurons to total LDH activity during 1 h after the exposure to 100 μM NMDA, 5 μM SNAP and 10 μM peroxynitrite for 5 min were 7.2 ± 1.6%, 5.1 ± 1.4% and 7.6 ± 1.8% (the means ± S.E.M. determined from 4 separate experiments), respectively. These ratios were not significantly different from that observed in the non-treated neurons (5.3 ± 1.1%, the mean ± S.E.M. determined from 4 separate experi-

Table 2
Effects of NMDA and peroxynitrite on [^{3}H]GABA release from cerebral cortical neurons

	[^{3}H]GABA release (dpm/mg protein/interval)	
	NMDA [a]	Peroxynitrite [b]
1st stimulation		
Fraction No. 3 (b_1)	4195	3500
4 (s_1)	7761	5719
S_1 (s_1/b_1)	1.85	1.63
2nd stimulation		
Fraction No. 8 (b_2)	3547	2989
9 (s_2)	6739	4685
S_2 (s_2/b_2)	1.90	1.57
Ratio (S_2/S_1)	1.02	0.96

After the neurons were preincubated with 2 nM [^{3}H]GABA (1.0 μCi [^{3}H]GABA/dish) in Krebs-Ringer bicarbonate buffer containing 20 mM Hepes (Hepes-KRB, pH 7.4) at 37°C for 30 min followed by washing of the cells three times with ice-cold Hepes-KRB, the neurons were preincubated in Hepes-KRB at 37°C for 10 min. Thereafter, the neurons were incubated in Hepes-KRB at 37°C at an interval of 5 min for a total of 50 min. When examining the effect of NMDA, Mg^{2+}-free Hepes-KRB was used as the incubation buffer to prevent the inhibitory actions of Mg^{2+} on functions of NMDA receptors. To investigate the effect of the agents, the agents were ordinarily added at the initiation of the 4th interval of the incubation. The 2nd stimulation (9th interval with agents) was carried out to check whether NMDA and peroxynitrite induced deterioration of the functional process of [^{3}H]GABA release. Each value represents the mean of 3 separate experiments, each of which was carried out in triplicate. The value of the S.E.M. is less than 10% of each mean value. [a] 100 μM, [b] 10 μM.

ments). Similarly, the exposure of the neurons to these agents did not affect the activity of the neurons to exclude trypan blue dye when compared with the activity of the non-treated neurons (data not shown). Based on these results, it is considered that the neurons maintain the functional process to release [^{3}H]GABA during the release experiments carried out in this study.

4. Discussion

In the present study, we attempted to clarify the participation of peroxynitrite formed by the reaction of NO with superoxide in NMDA- and SNAP-induced release of [^{3}H]GABA from primary cultured cerebral cortical neurons and to investigate the mechanisms for the release of [^{3}H]GABA evoked by peroxynitrite.

Although NMDA has been reported to evoke the release of GABA (Drejer et al., 1987; Pin et al., 1988), it is also known to show neurotoxicity. Peroxynitrite has been reported to exert several biochemical effects on cell functions through its potent oxidizing property (Beckman et al., 1990; Hogg et al., 1992; Ischiropoulos et al., 1992a; Radi et al., 1991a) and neurotoxicity (Lipton et al., 1993). Accordingly, we have carried out a preliminary experiment to evaluate whether agents such as NMDA and peroxynitrite, which have the ability to evoke the release of [^{3}H]GABA, deteriorate the functions of neuronal membrane during the experiments to investigate pharmacological properties on [^{3}H]GABA release evoked by these agents. As shown in Table 2, the ratios of S_2 to S_1 are almost 1.0. In addition, NMDA, peroxynitrite and SNAP showed no effects on the leakage of LDH activity from the neurons and on the activity of the neurons to exclude trypan blue dye. These data are considered to support that none of these agents deteriorate the membrane functions including processes relating to GABA release, at least, during the experimental period employed here.

Since the stimulation of NMDA receptors induces the increase in NO synthase activity to form NO, the possibility that the NMDA-induced release of GABA may be due to the NO formation facilitated by the NMDA receptor activation is assumed. In fact, the NMDA-induced [^{3}H]GABA release found in this study was almost abolished by NO synthase inhibitors. These data reveal the essential role of NO in the release of [^{3}H]GABA induced by the NMDA receptor activation. Similarly, the role of NO in NMDA-induced neurotransmitter release has been reported (Hanbauer et al., 1992; Montague et al., 1994; Ohkuma et al., 1995a).

The release of [^{3}H]GABA induced by the NMDA receptor stimulation has been abolished by hemoglobin as presented here. This is considered to be similar to the case of NMDA-induced releases of dopamine (Hanbauer et al., 1992) and acetylcholine (Montague et al., 1994; Ohkuma et al., 1995a). These results suggest that NO produced by the NMDA receptor stimulation may initially diffuse out through neuronal membrane to the extracellular space and then act extracellularly on neurons to evoke the release of neurotransmitters, because it is not likely that hemoglobin with a molecular weight of approximately 60 kDa is incorporated into the neurons, and consequently scavenges NO formed in neuronal cytoplasm during the short incubation period (5 min) used in this study.

One of the new findings demonstrated here is that SOD suppresses the NMDA-induced increase in [^{3}H]GABA release. Another superoxide scavenger, ceruloplasmin, also induced the reduction in the release of [^{3}H]GABA enhanced by NMDA. In addition, [^{3}H]GABA release by SNAP was also inhibited by these superoxide scavengers (Ohkuma et al., 1995b). These inhibitory effects of superoxide scavengers on the increased release of [^{3}H]GABA induced by NO indicate that NO requires superoxide to induce [^{3}H]GABA release. These results raise the possibility that products formed from both NO and superoxide may be involved in the release of [^{3}H]GABA induced by both NMDA and SNAP. As previously reported, NO reacts rapidly with superoxide to form peroxynitrite in vivo (Blough and Zafiriou, 1985; Ischiropoulos et al., 1992a). Therefore, it is supposed that the increase in [^{3}H]GABA release induced by SNAP and NMDA is due

to peroxynitrite, which is extracellularly produced by the reaction of superoxide with NO. In addition, SOD has the ability to scavenge not only superoxide but peroxynitrite (Ischiropoulos et al., 1992b), which is considered to result in the disappearance of the stimulatory effect of NO on [^{3}H]GABA release. However, this event seems to be unlikely, because the addition of SOD into the experimental system is performed before the formation of NO and, therefore, superoxide may be removed before the reaction to form peroxynitrite.

Peroxynitrite formed under the conditions at pH 7.4 is unstable ($T_{1/2} < 1.9$ s) (Beckman et al., 1990). In spite of its short half-life under physiological conditions, it alters cellular functions. That is, peroxynitrite has been reported to change the membrane permeability to sodium ions (Bauer et al., 1992), and to have bactericidal activity (Zhu et al., 1992). Moreover, we have demonstrated that the direct addition of synthesized peroxynitrite into the incubation buffer triggers the dose-dependent increase in [^{3}H]GABA release from the cerebral cortical neurons (Ohkuma et al., 1995b). In addition, peroxynitrite exhibits potent oxidizing effects on intracellular components such as protein thiols (Radi et al., 1991a), deoxyribose (Beckman et al., 1990) and membrane phospholipid (Radi et al., 1991b). Taken these data together, it is assumed that peroxynitrite is formed extracellularly by the reaction of NO and superoxide and then alters the release process by modifying functions of molecules participating in the process of neurotransmitter release.

Recent investigations reveal that biological effects induced by peroxynitrite are partially mediated by NO generated from the molecule of peroxynitrite (Moro et al., 1994). The stimulatory action of peroxynitrite on [^{3}H]GABA release observed in this study and the previous report (Ohkuma et al., 1995b) may therefore be due to NO liberated from peroxynitrite, although whether such an event occurs is not clear at present. In addition, we have demonstrated that hemoglobin abolishes the stimulatory effects of the NMDA receptor stimulation as presented here and of SNAP (Ohkuma et al., 1995b) on [^{3}H]GABA release, and that such NO-induced [^{3}H]GABA release is due to peroxynitrite formed from NO and superoxide. However, it has been reported that hemoglobin has the ability to scavenge both NO and peroxynitrite (Schmidt et al., 1994). These data raise a possibility that the disappearance of the stimulatory effect of NO on [^{3}H]GABA release in the presence of hemoglobin may be partially attributed to the effect of hemoglobin to scavenge both peroxynitrite and NO.

It is noteworthy that the SNAP-evoked [^{3}H]GABA release occurs through two different release mechanisms as demonstrated in the present study. That is, the SNAP-evoked release of [^{3}H]GABA is operated by Ca^{2+}-dependent and Ca^{2+}-independent release systems. The latter system is considered to be the reverse process of a Na^+-dependent carrier-mediated GABA transport system, because [^{3}H]GABA release induced by SNAP is dose dependently inhibited by two inhibitors of the Na^+-dependent carrier-mediated GABA transport system, nipecotic acid and NO-711 (Dong et al., 1984), either in the presence or absence of Ca^{2+} and these inhibitors reduced the SNAP-induced release to the basal level in the absence of Ca^{2+}. Although there are no reports mentioning the involvement of the reverse process of a Na^+-dependent carrier-mediated GABA transport system in NO-induced [^{3}H]GABA release at present, similar mechanisms of GABA release, i.e. the reverse process of a Na^+-dependent carrier-mediated GABA transport system, from neuronal growth cones of the forebrain (Taylor and Gordon-Weeks, 1991), striatal neurons (Pin and Bockaert, 1989) by high K^+ and striatal slice by electrical field stimulation (Bernath and Zigmond, 1988) have been reported. On the other hand, Guevara-Guzman et al. (1994) have reported Ca^{2+} dependency of GABA release induced by NO, which is considered to be consistent with the data reported here that the removal of Ca^{2+} reduced significantly the SNAP-evoked [^{3}H]GABA release.

In the present study we have revealed that a part of the NMDA-evoked [^{3}H]GABA release is mediated by a reverse process of the Na^+-dependent carrier-mediated GABA transport system, whereas the Ca^{2+}-dependent release system also participates in the release of [^{3}H]GABA by NMDA. This conclusion is induced by the data that a part of the NMDA-evoked [^{3}H]GABA release is inhibited significantly by GABA transport inhibitors and a remaining part of the release is reduced by the inhibitors of voltage-dependent L-typed Ca^{2+} channel, verapamil and nifedipine, to the basal release as shown in this study. We have already reported that NMDA induced Ca^{2+} entry into the cerebral cortical neurons through both Ca^{2+} ionophore coupling with NMDA receptors and voltage-dependent L-typed Ca^{2+} channels (Ohkuma et al., 1994a). Therefore, this Ca^{2+}-dependent GABA release induced by NMDA may be operated by Ca^{2+} flowing into the neurons subsequent to the opening of voltage-dependent L-typed Ca^{2+} channels by NMDA. However, several investigators reported that GABA release induced by NMDA is mediated via only a reverse process of the Na^+-dependent carrier-mediated GABA transport system (Pin and Bockaert, 1989; Belhage et al., 1993). At present, the reasons of such discrepancy in the involvement of Ca^{2+}-dependent release of GABA evoked by NMDA are not clear.

One of the important and new findings demonstrated in this study is that peroxynitrite also releases [^{3}H]GABA from the neurons via both Ca^{2+}-dependent and Ca^{2+}-independent release systems. The latter system is assumed to be a reverse process of the Na^+-dependent carrier-mediated GABA transport system, which may be supported by the data that nipecotic acid and NO-711 inhibited dose dependently the peroxynitrite-evoked [^{3}H]GABA release under the conditions with and without Ca^{2+}. Moreover, the withdrawal of Ca^{2+} from the incubation buffer reduced the

peroxynitrite-induced [^{3}H]GABA release by about 50%. When comparing these patterns of the peroxynitrite-evoked [^{3}H]GABA release with those of the releases of [^{3}H]GABA by SNAP and NMDA, the mechanisms of peroxynitrite to induce [^{3}H]GABA release is considered to be similar to those of NO described above. These data, therefore, may further confirm that peroxynitrite is involved in NO-evoked [^{3}H]GABA release.

In summary, we have investigated the role of peroxynitrite in NMDA- and SNAP-induced [^{3}H]GABA release from primary cultured cerebral cortical neurons and the mechanisms of peroxynitrite to release [^{3}H]GABA in comparison with those of SNAP and NMDA. NMDA dose dependently increased the release of [^{3}H]GABA, which disappeared in the presence of NO synthase inhibitors. The increase in [^{3}H]GABA release by the NMDA receptor stimulation was completely abolished by hemoglobin and superoxide scavengers such as SOD and ceruloplasmin. Similar inhibition of the SNAP-evoked [^{3}H]GABA release by these superoxide scavengers was reported. These results suggest the possible involvement of peroxynitrite formed by the reaction of NO with superoxide in NO-induced [^{3}H]GABA release. Indeed, the stimulation of [^{3}H]GABA release by synthesized peroxynitrite has been reported. Therefore, it is presumed that the enhancement of GABA release by NO is, in part, due to peroxynitrite, a product formed by the reaction of NO with superoxide. The removal of Ca^{2+} reduced the SNAP-induced [^{3}H]GABA release by about 50%. Two GABA transport inhibitors, nipecotic acid and NO-711, suppressed dose dependently the SNAP-induced [^{3}H]GABA release either in the presence or absence of Ca^{2+} and these inhibitors abolished completely the stimulatory effect of SNAP in the absence of Ca^{2+}. These results suggest that NO liberated from SNAP releases [^{3}H]GABA through both a Ca^{2+}-dependent release system and a reverse process of the Na^+-dependent carrier-mediated GABA transport system. Similar release patterns were observed in cases of the NMDA- and peroxynitrite-evoked [^{3}H]GABA releases. These data on the mechanisms for [^{3}H]GABA release are also considered to confirm the participation of peroxynitrite in NO-evoked [^{3}H]GABA release.

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