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Involvement of Hg^{2+}-Sensitive Sulfhydryl Groups in Regulating Noradrenaline Release Induced by *S*-Nitrosocysteine in Rat Brain Slices

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ABSTRACT. Nitric oxide has been shown to regulate neurotransmitter release. Previously, we reported that *S*-nitrosothiols such as *S*-nitrosocysteine (SNC) stimulate noradrenaline (NA) release in rat hippocampus *in vivo* and *in vitro*. To examine the role of sulfhydryl groups in SNC-induced NA release, the effects of metal ions such as Hg^{2+} and *N*-ethylmaleimide (NEM, a sulfhydryl alkylating agent) on [^{3}H]NA release from labeled rat brain slices (hippocampus and cerebral cortex) were studied and compared with the effects of SNC. The addition of 200 μM $HgCl_2$, but not Pb^{2+}, Zn^{2+}, or Cd^{2+}, stimulated [^{3}H]NA release from both types of slices in the presence of extracellular $CaCl_2$. *p*-Chloromercuribenzoic acid (*p*-CMBA) also stimulated [^{3}H]NA release. NEM stimulated [^{3}H]NA release from both types of slices in the presence and absence of extracellular $CaCl_2$. The effect of 200 μM NEM was enhanced, but the effect of 200 μM SNC was inhibited by co-addition of 200 μM *p*-CMBA in the absence of extracellular $CaCl_2$. The concentration–response curve of SNC shifted to the right after co-addition of 200 μM *p*-CMBA or 100 μM $HgCl_2$, although the effect of 200 μM NEM was additive to the effect of SNC. These findings demonstrate that SNC acts as a sulfhydryl agent on proteins that regulate NA release, and that SNC may share the same sulfhydryl groups with Hg compounds. The effect of T-588 {(*R*)-(-)-(benzo[*b*]thiophen-5-yl)-2-[2-(*N*,*N*-diethylamino)ethoxy]ethanol hydrochloride}, a novel cognitive enhancer and a stimulator of NA release, was compared with the effects of sulfhydryl reagents. BIOCHEM PHARMACOL **59**;7:839–845, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. noradrenaline release; sulfhydryl reagent; $HgCl_2$; *N*-ethylmaleimide; *S*-nitrosocysteine; brain slices

Metal ions have various effects on the central nervous system. Hg is selectively concentrated in human brain regions, including the amygdala and the hippocampus, which are involved with memory. Post-mortem analysis of human brains reveals that inorganic Hg concentrations are higher in the amygdala and hippocampus in Alzheimer's disease brains compared with age-matched control brains [1]. Pb exposure leads to mental retardation, cognitive impairments, and maladaptive behavior. High Pb blood levels (>10 μg/dL) are associated with deficits in learning and memory [2, 3]. Since alterations in neurotransmitter release are believed to underlie the processes of learning and memory (for reviews, see Refs. 4 and 5), it is possible that metal ions may affect neurotransmitter release from neuronal tissues.

Ca^{2+} plays an important role in neurotransmitter release. Several metal ions including Hg^{2+} block Ca^{2+} channels and inhibit transmitter release (see Introductions in Refs. 6 and 7). On the other hand, metal ions including Hg^{2+} and organomercurial agents regulate Ca^{2+} release from the sarcoplasmic reticulum [8] and intracellular Ca^{2+} pools in various tissues, including neuronal tissues [9–11]. Thus, metal ions such as Hg^{2+} may regulate neurotransmitter release via control of $[Ca^{2+}]_i$,‡ but the effects of metal ions on Ca^{2+} release and on other factors regulating neurotransmitter release have not been investigated thoroughly.

There have been several reports on the effects of NEM, a sulfhydryl alkylating agent, on neurotransmitter release. For instance, NEM at low concentrations (50–100 μM) enhances acetylcholine-stimulated catecholamine release from adrenal medulla and chromaffin cells [12, 13]. Wu *et al.* [14] found that NEM enhances Ca^{2+}-dependent NA secretion from permeabilized chromaffin cells. In rat hippocampal slices, NEM significantly enhanced [^{3}H]glutamate and [^{3}H]NA release, probably via modification of GTP-binding proteins [15, 16]. Previously, we reported that

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‡ *Abbreviations*: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentrations; *p*-CMBA, *p*-chloromercuribenzoic acid; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNC, *S*-nitrosocysteine; NA, noradrenaline; and SNP, sodium nitroprusside.

(*R*)-(-)-(benzo[*b*]thiophen-5-yl)-2-[2-(*N*,*N*-diethylamino) ethoxy]ethanol hydrochloride (T-588), a novel cognitive enhancer [17–19], stimulates NA release by itself, but inhibits NEM-stimulated NA release [20]. Although NEM is proposed to inhibit vesicle transport via NEM-sensitive fusion protein [21, 22], NEM stimulates NA release in several cell types as described above. Thus, other proteins that have a sulfhydryl group(s) and regulate transmitter release are also possible targets.

In neuronal tissues, NO donors have been reported to modulate secretion of hormones and neurotransmitters such as NA, acetylcholine, and dopamine (for a review, see the Introduction in Ref. 23). In rat hippocampal slices preincubated with [^{3}H]NA, [^{3}H]NA release was stimulated by endogenous NO [24] and NO donors [25]. Previously, we reported that NO donors including SNP and SNAP in the presence of cysteine, and SNC by itself, stimulate [^{3}H]NA release in a cyclic GMP-independent manner from labeled rat hippocampal slices [23, 26, 27]. SNC-stimulated [^{3}H]NA release from hippocampal slices [27] and NO donor-stimulated synaptic vesicle release from hippocampal synaptosomes [28] are independent of extracellular $CaCl_2$ and a rise in $[Ca^{2+}]_i$, respectively. Several NO species including SNC react with cysteine residues and *S*-nitrosylate proteins [29]. These findings demonstrate that modification of sulfhydryl groups on proteins by NO donors is involved in the regulation of NA release from neuronal tissues, but Ca^{2+} channels are not. In this study, we investigated (i) the effects of various metal ions on [^{3}H]NA release from labeled rat hippocampal and cerebral cortical slices, (ii) the effects of SNC and NEM on release, and (iii) the difference between the effects of sulfhydryl reagents and Hg compounds. The results showed that SNC may share the same sulfhydryl groups with Hg compounds and that it stimulates NA release.

MATERIALS AND METHODS

Materials

L-[7,8-^{3}H]NA (1.44 TBq/mmol) was purchased from Amersham. DTT and NEM were purchased from the Sigma Chemical Co. *p*-CMBA, SNAP, SNP, and other materials were obtained from Wako Pure Chemical Industries. SNC was a gift from Dr. Miyazaki (Dojindo Lab.). T-588 was synthesized by the Toyama Chemical Co. Ltd.

Preparation of Rat Cerebral Cortical and Hippocampal Slices

Male Wistar rats (Japan Laboratory Animals Inc.) weighing 150–300 g were used. They were housed at constant temperature and controlled illumination (light on between 7:30 a.m. and 7:30 p.m.). Food and water were available *ad lib*. All animal experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at Hokkaido University. We used decapitation instead of deep anesthesia to avoid the effects of anesthetics. The hippocampi from two rats and the cerebral cortex from one rat were generally used for each experiment. The slices were prepared according to a method described previously [20, 23, 26, 27, 30]. The slices were washed twice with Tyrode's HEPES buffer (composition: 134 mM NaCl, 3 mM KCl, 1 mM Na_2HPO_4, 12 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 20 mM HEPES, pH 7.0) followed by centrifugation at 4° (100 *g*, 15 sec). The Tyrode's buffer was oxygenated prior to use by bubbling with O_2 gas.

Measurement of [^{3}H]NA Release

Slice suspensions were labeled with 50 nM L-[7,8-^{3}H]NA for 30 min at 37° in Tyrode's buffer (pH 7.2) containing 10 μM nialamide (monoamine oxidase inhibitor), 20 μg/mL of aprotinin (protease inhibitor), and 0.3 mM phenylmethylsulfonyl fluoride (serine protease inhibitor). The pH of the buffer used for the labeling reaction was lowered (to 7.0) to avoid NA degradation. In some experiments, slice suspensions were labeled with [^{3}H]NA in the indicated Tyrode's buffer supplemented with 10 μM nomifensine, an inhibitor of dopamine transport. Because data from both procedures were similar, [^{3}H]NA seemed to be incorporated via NA transporters. Labeled slices were washed three times with ice-cold Tyrode's buffer (pH 7.4) by centrifugation at 4° (100 *g*, 15 sec) to remove extracellular [^{3}H]NA. Labeled and washed slices (100–200 μg protein/tube) were incubated for 8 min at 37° in modified Tyrode's buffer (total volume, 0.3 mL) containing 0.2% bovine serum albumin with or without test compounds. In some experiments, slices were preincubated with DTT for 10 min at 37°. The release reaction was terminated by the addition of 0.5 mL of ice-cold HEPES buffer (composition: 137 mM NaCl, 5 mM EGTA, 5 mM EDTA, 20 mM HEPES, pH 7.4) followed by centrifugation at 4° (1000 *g*, 15 sec). The radioactivity in the supernatant was quantitated with a liquid scintillation spectrometer. This procedure for measuring [^{3}H]NA release has been used frequently for neuronal tissues [31, 32], and increases in tritium induced by electrical field stimulation have been shown to be composed predominantly of intact NA [33]. Lonart and Johnson [25] reported that over 90% of the tritium released by SNC from hippocampal slices labeled with [^{3}H]NA is intact [^{3}H]NA. In this study, there were no differences in basal (unstimulated) and agent-stimulated [^{3}H]NA release in the presence or absence of 2 μM nialamide in the assay mixture. Data are presented as percentages of total incorporated [^{3}H]NA. Protein content was determined with a Bio-Rad assay kit using bovine serum albumin as a standard.

Statistics

Data are reported as means ± SEM. In the case of multiple comparisons, significance of differences was determined by ANOVA followed by Dunnett's or Tukey's test. In Fig. 1,

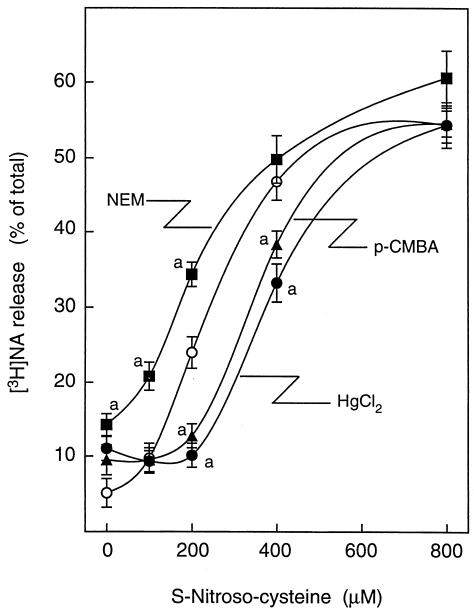

FIG. 1. [^{3}H]NA release from rat cerebral cortical slices induced by SNC. Labeled cerebral cortical slices were incubated with the indicated concentrations of SNC in the absence of extracellular $CaCl_2$. The assay mixture was supplemented further with vehicle (○), 200 μM NEM (■), 200 μM *p*-CMBA (▲), or 100 μM $HgCl_2$ (●). The concentrations of methanol (the solvent of the SNC solution) in assay mixtures were under 3%, and up to 5% methanol had no effect on [^{3}H]NA release. Results are expressed as means ± SEM of 3–4 independent experiments. The slope parameters were 0.12 + 0.01 (■), 0.14 + 0.01 (▲), and 0.12 + 0.01 (●), which were the same as 0.12 + 0.01 (○) in SNC alone. Key: (a) $P < 0.01$ vs the effect by SNC alone.

EC_{50} values were calculated by a computerized nonlinear regression program, the SAS system (Ver. 3, SAS Institute Inc.). A probability of $P < 0.01$ was considered to be significant. In some experiments, data are reported as means ± SD of three determinations in a typical experiment, and data are representative of two independent experiments.

RESULTS

Effects of Metal Ions on [^{3}H]NA Release

Previously, we reported that SNC stimulates endogenous NA release from rat hippocampus *in vivo* and [^{3}H]NA release from prelabeled hippocampal slices [27]. To investigate the mechanism of SNC-induced release, we used an *in vitro* assay system in this study. First, we examined the effects of various divalent metal ions on [^{3}H]NA release from labeled rat hippocampal slices in the presence of 1 mM extracellular $CaCl_2$ (Table 1). The addition of 200 μM $HgCl_2$ remarkably stimulated, while $ZnCl_2$ and $NiCl_2$ only slightly stimulated, [^{3}H]NA release. Other metal ions slightly inhibited [^{3}H]NA release. The addition of 100 μM $HgCl_2$ slightly stimulated [^{3}H]NA release (17.3 ± 3.4%), but other metal ions at this concentration showed no effect. In the case of cerebral cortical slices, similar results were found (data not shown). Next, we investigated the effect of

TABLE 1. [^{3}H]NA release from rat hippocampal slices by divalent cations

Metals (200 μM)	[^{3}H]NA release (% of total)
None	8.1 ± 2.3
$HgCl_2$	40.1 ± 3.2
$FeCl_2$	5.2 ± 0.1
$PbCl_2$	6.8 ± 1.3
$ZnCl_2$	12.4 ± 0.8
$CdCl_2$	4.8 ± 0.5
$NiCl_2$	10.3 ± 0.5
$MnCl_2$	6.1 ± 2.2

Labeled hippocampal slices were incubated with 200 μM of various divalent metals in the presence of 1 mM $CaCl_2$. Results are means ± SD of 3 determinations in a typical experiment. Results are typical of 2 independent experiments.

1 mM extracellular $CaCl_2$ on [^{3}H]NA release induced by $HgCl_2$ from hippocampal and cerebral cortical slices (Table 2). In the absence of $CaCl_2$, $HgCl_2$ significantly stimulated [^{3}H]NA release from cerebral cortical slices. $HgCl_2$ also stimulated [^{3}H]NA release from hippocampal slices, although the effect was not significant because of large variations in the data. Net increases in [^{3}H]NA release induced by 200 μM $HgCl_2$ from both types of slices in the presence of $CaCl_2$ were much stronger than in the absence of $CaCl_2$. In cerebral cortical slices, the effect of 100 μM $HgCl_2$ in the presence or absence of extracellular $CaCl_2$ was 18.4 ± 2.1 and 10.8 ± 1.8% (N = 3), respectively. Because adding 0.5 μM tetrodotoxin, a voltage-dependent Na^+ channel inhibitor, had no effect on [^{3}H]NA release induced by 200 μM $HgCl_2$ from either type of brain slices (data not shown), Na^+ channel activity was not involved in the $HgCl_2$ effect. [^{3}H]NA release induced by $HgCl_2$ was not the result of cell toxicity, because lactate dehydrogenase leakage in the supernatant from $HgCl_2$-treated slices (200 and 400 μM, for 8 min) was the same as that from

TABLE 2. Extracellular Ca^{2+}-dependent and -independent [^{3}H]NA release from rat brain slices

	[^{3}H]NA release (% of total)	
Addition	$CaCl_2$-free	1 mM $CaCl_2$
Experiment I: in hippocampal slices		
None	5.1 ± 2.0	8.1 ± 1.1
$HgCl_2$ (200 μM)	10.6 ± 1.0	40.7 ± 1.9*†
NEM (200 μM)	34.2 ± 4.4†	29.6 ± 2.5†
SNC (500 μM)	35.3 ± 3.4†	38.3 ± 4.2†
Experiment II: in cerebral cortical slices		
None	4.4 ± 0.6	11.2 ± 1.1*
$HgCl_2$ (200 μM)	13.9 ± 2.1†	34.8 ± 1.2*†
p-CMBA (200 μM)	10.9 ± 0.1†	23.0 ± 0.2*†
NEM (400 μM)	32.0 ± 2.1†	29.5 ± 1.0†
SNC (400 μM)	39.7 ± 0.5†	46.6 ± 1.3†
T-588 (3 mM)	32.4 ± 1.1†	31.9 ± 1.4†

Labeled hippocampal (Experiment I) and cerebral cortical (Experiment II) slices were incubated with the indicated agents in the presence or absence of 1 mM extracellular $CaCl_2$. EGTA (0.2 mM) was further added to the $CaCl_2$-free buffer. Results are means ± SEM of 3–4 independent experiments.

*$P < 0.01$ vs effect without $CaCl_2$.

†$P < 0.01$ vs none in the presence or absence of $CaCl_2$.

TABLE 3. Effects of DTT on [^{3}H]NA release from rat cerebral cortical slices

Addition	[^{3}H]NA release (% of total)	
	None	5 mM DTT
None	13.3 ± 1.7	12.3 ± 0.5
$HgCl_2$ (200 μM)	35.0 ± 1.7*	13.1 ± 0.8†
p-CMBA (200 μM)	29.9 ± 0.9*	18.6 ± 0.8*†
NEM (400 μM)	31.0 ± 1.2*	12.7 ± 0.4†
SNC (400 μM)	45.5 ± 2.6*	21.5 ± 2.4*†
T-588 (3 mM)	28.1 ± 1.0*	26.4 ± 2.2*

Labeled cerebral cortical slices were incubated with the indicated concentrations of agents in the presence or absence of 5 mM DTT. The assay mixture was further supplemented with 1 mM $CaCl_2$. Results are means ± SEM of 3–4 independent experiments.

*$P < 0.01$ vs none in the presence or absence of DTT.

†$P < 0.01$ vs effect without DTT.

untreated slices (<2% of the total activity in the supernatant).

Effects of Sulfhydryl Agents on [^{3}H]NA Release from Rat Cerebral Cortical Slices

Some metal ions including Hg^{2+} act as sulfhydryl reagents in muscle [8]. We investigated the effects of *p*-CMBA (another sulfhydryl reagent), NEM (a sulfhydryl alkylating agent), and SNC on brain slices (Table 2). The addition of *p*-CMBA (200 μM) stimulated [^{3}H]NA release from prelabeled rat cerebral cortical slices slightly, but significantly, in the absence of $CaCl_2$. In the presence of $CaCl_2$, the effect of *p*-CMBA was marked. The addition of the indicated concentrations of NEM stimulated [^{3}H]NA release significantly and independently of extracellular $CaCl_2$ from both types of slices. The effect of SNC on [^{3}H]NA release from cerebral cortical slices in the absence of extracellular $CaCl_2$ was the same as that in the presence of $CaCl_2$, as shown in hippocampal slices (Table 2; [26]). Thus, the effect of NEM and SNC was independent of $CaCl_2$ addition in both brain slice types. These findings suggest that Hg^{2+}, NEM, and SNC stimulated [^{3}H]NA release from both types of slices in a similar manner. For further analysis of NA release induced by sulfhydryl agents, we used rat cerebral cortical slices in the following experiments.

Table 3 shows the effect of 5 mM DTT on [^{3}H]NA release from prelabeled rat cerebral cortical slices. In this series of experiments, the slices were incubated with or without 5 mM DTT for 10 min, and the washed slices were incubated with the indicated agents for 8 min in the presence or absence of 5 mM DTT. The effect of 200 μM $HgCl_2$ was inhibited completely by DTT treatment, and the effect of 200 μM *p*-CMBA was inhibited significantly. Treatment with DTT by itself had no effect on [^{3}H]NA release. The effect of NEM also was inhibited completely. DTT partially, but significantly, inhibited the effect of 400 μM SNC.

Interactions Between Sulfhydryl Groups

Figure 1 shows the effect of SNC on [^{3}H]NA release in the absence of extracellular $CaCl_2$ from rat cerebral cortical slices. The addition of SNC to the assay mixture stimulated [^{3}H]NA release in a concentration-dependent manner with maximal effect at 800 μM. Over this concentration range, [^{3}H]NA release decreased because of inhibition by the solvent of the SNC solution (SNC was dissolved in methanol to avoid decay). The EC_{50} value was 254 ± 8 μM (N = 4). The addition of 100 μM $HgCl_2$ or 200 μM *p*-CMBA, which slightly stimulated [^{3}H]NA release in the absence of $CaCl_2$, shifted the concentration-dependence of SNC and significantly inhibited [^{3}H]NA release induced by 200 or 400 μM SNC; the EC_{50} values were 408 ± 27 (N = 4) and 354 ± 12 μM (N = 4) in the presence of 100 μM $HgCl_2$ or 200 μM *p*-CMBA, respectively. [^{3}H]NA release induced by 800 μM SNC was not inhibited. The addition of 100 or 200 μM SNC to 200 μM NEM, which significantly stimulates [^{3}H]NA release by itself in the absence of $CaCl_2$, had an additive effect. The EC_{50} value of SNC was 241 ± 5 μM in the presence of 200 μM NEM (N = 3). Table 4 shows the effects of co-addition of *p*-CMBA and NEM or SNC in the absence of extracellular $CaCl_2$. The addition of 200 μM *p*-CMBA stimulated [^{3}H]NA release slightly, but significantly, and enhanced the effect of 200 μM NEM. The effect of 400 μM NEM and 200 μM *p*-CMBA was additive. On the other hand, [^{3}H]NA release induced by 200 μM SNC was inhibited significantly by *p*-CMBA; [^{3}H]NA release stimulated by co-addition of 200 μM *p*-CMBA and 200 μM SNC was nearly equal to that of *p*-CMBA itself (Table 4 and Fig. 1). The co-addition of 100 μM SNC and 200 μM *p*-CMBA did not have an additive effect.

These results suggested that $HgCl_2$ and *p*-CMBA reacted with sulfhydryl groups in proteins; therefore, we investigated the effect of pretreatment with *p*-CMBA. Slices were incubated with 100 μM *p*-CMBA for 5 min at 37° in the absence of extracellular $CaCl_2$, washed, and [^{3}H]NA release was assayed in the absence of $CaCl_2$. The basal

TABLE 4. Effects of *p*-CMBA on [^{3}H]NA release from rat cerebral cortical slices

Addition	[^{3}H]NA release (% of total)	
	None	200 μM *p*-CMBA
None	4.4 ± 0.9	8.5 ± 1.1*
NEM (200 μM)	12.4 ± 1.1	23.2 ± 2.1*
(400 μM)	29.9 ± 2.2	36.0 ± 2.0
SNC (100 μM)	9.8 ± 1.5	9.6 ± 1.2
(200 μM)	24.5 ± 1.7	12.4 ± 1.2*
T-588 (1 mM)	17.0 ± 1.2	21.3 ± 0.8
(3 mM)	32.3 ± 1.4	35.8 ± 1.6

Labeled cerebral cortical slices were incubated with the indicated concentrations of agents in the absence of extracellular $CaCl_2$. The assay mixture was further supplemented with or without 200 μM *p*-CMBA. Results are means ± SEM of 3–4 independent experiments.

*$P < 0.01$ vs effect without *p*-CMBA.

(unstimulated) release was 4.9 ± 0.5 and 11.6 ± 0.3% (means ± SD of a typical experiment) from untreated and *p*-CMBA-treated slices, respectively. In these conditions, [^{3}H]NA release induced by 200 μM NEM was enhanced 2-fold, and the effect of 200 μM SNC was inhibited by about 50% in *p*-CMBA-treated slices. These findings suggested that the effect of *p*-CMBA is not washed out, and that *p*-CMBA sensitivity is different for NEM and SNC. SNC, but not NEM, may modify the same sulfhydryl group(s) in protein(s) as do $HgCl_2$ and *p*-CMBA.

Effects of T-588 on [^{3}H]NA Release

T-588 protects against cerebral hypoxia, anoxia, and ischemia [17], and partially inhibits memory and learning impairments [19]. T-588 at millimolar concentrations stimulates NA release from rat cerebral cortical slices [20, 30]. Because T-588 inhibits [^{3}H]NA release induced by 600 μM NEM [20], T-588 may interact with sulfhydryl groups. Thus, we compared the effects of T-588 with those of $HgCl_2$ and SNC in this study. As previously reported [20], the addition of 3 mM T-588 stimulated [^{3}H]NA release in the presence and absence of $CaCl_2$, and the effect of T-588 was not modified by treatment with 5 mM DTT (Tables 2 and 3). Further, the effect of T-588 was not modified by co-addition of 200 μM *p*-CMBA (Table 4), and pretreatment with 100 μM *p*-CMBA did not modify the effects of 1 or 3 mM T-588 (data not shown).

DISCUSSION

NA Release Induced by $HgCl_2$ and the Role of Extracellular $CaCl_2$

Metal ions have many biological effects on the nervous system. For instance, metal ions such as Ni^{2+} and Cd^{2+} are potent blockers of Ca^{2+} channels and inhibit transmitter release [6, 7]. Hg^{2+} and methyl-Hg inhibit various types of Ca^{2+} channels in PC12 cells [34, 35]. In addition, several studies have suggested that some metal ions can interfere with the function of receptors or enzymes involved in second messenger systems (for a review, see the Introduction in Ref. 36) and can affect exocytosis at an intracellular locus [6, 37]. To understand the biological effects of metal ions further, we examined in rat brain slices [^{3}H]NA releases induced by metal ions. In brain slices, the addition of $HgCl_2$ (200 μM) significantly stimulated [^{3}H]NA release from rat hippocampal (Table 1) and cerebral cortical (Table 2) slices. Neither $PbCl_2$, $CdCl_2$, nor $NiCl_2$ had a significant effect on [^{3}H]NA release from rat hippocampal (Table 1) or cerebral cortical (data not shown) slices. Other metal ions used had no or very limited effects. $HgCl_2$-stimulated [^{3}H]NA release was not due to cell toxicity because (i) cell viability did not change following $HgCl_2$ addition, (ii) the effect of $HgCl_2$ was not observed in the presence of DTT (Table 3), and (iii) the effect of 100 μM $HgCl_2$, but not *p*-CMBA, on [^{3}H]NA release was abolished after washing with buffer (data not shown).

The addition of $HgCl_2$ and *p*-CMBA stimulated [^{3}H]NA release from cerebral cortical slices slightly, but significantly, in the absence of $CaCl_2$ (Table 2). Heavy metal ions such as Hg^{2+} and organomercury compounds act as sulfhydryl reagents and trigger Ca^{2+} release from intracellular Ca^{2+} pools in several cell types [8–11]. Thus, it is probable that $HgCl_2$ and related compounds induce $[Ca^{2+}]_i$ increases from intracellular Ca^{2+} pools and at least partially stimulate [^{3}H]NA release in brain slices. Tachikawa *et al.* [13] reported that *p*-CMBA causes Ca^{2+} influx and stimulates catecholamine release from cultured bovine adrenal medullary cells. In our experiments, [^{3}H]NA release induced by $HgCl_2$ (and *p*-CMBA) from both brain slice types in the presence of extracellular $CaCl_2$ was much higher than in the absence of $CaCl_2$ (Table 2). Although the effect on NA release of the contribution to $[Ca^{2+}]_i$ increases from extracellular and intracellular Ca^{2+} pools by $HgCl_2$ and *p*-CMBA has not been clarified, Hg^{2+} and the related compounds regulate Ca^{2+} mobilization and stimulate NA release in brain slices.

Effects of SNC, p-CMBA, and NEM on NA Release

As reported previously in the hippocampus [26], the addition of SNC also stimulated NA release from hippocampal and cerebral cortical slices in an extracellular $CaCl_2$-independent manner (Table 2). NO compounds, including SNC, react with cysteine residues and *S*-nitrosylate proteins [29]. NO donors induce *S*-nitrosylation of sulfhydryl groups on neighboring (vicinal) cysteine residues in the *N*-methyl-D-aspartate (NMDA) receptor/channel [38], and SNC and NEM regulate cyclic nucleotide-gated channels in olfactory neurons [39]. Recently Xu *et al.* [40] reported that the ryanodine receptor activates Ca^{2+} channel activity after *S*-nitrosylation by SNC or alkylation by NEM. In PC12 cells, SNC stimulates Ca^{2+} mobilization from caffeine-sensitive Ca^{2+} pools regulated by ryanodine receptors [41]. As mentioned above, Hg^{2+} and related compounds induce $[Ca^{2+}]_i$ increases from intracellular Ca^{2+} pools [8, 9]. These reports suggest that SNC, NEM, and Hg compounds react with sulfhydryl groups of protein(s) that form or regulate Ca^{2+} channels. In our experiments, the addition of 200 μM *p*-CMBA stimulated [^{3}H]NA release from the cerebral cortical slices in the absence of extracellular $CaCl_2$, similarly to $HgCl_2$ (Table 2), and NEM stimulated [^{3}H]NA release from both slice preparations in an extracellular $CaCl_2$-independent manner. The effects of 400 μM NEM or 200 μM *p*-CMBA were abolished completely or almost completely, respectively, by the co-addition of 5 mM DTT. Our findings indicate a possibility that sulfhydryl reagents such as Hg compounds interact with intracellular Ca^{2+} pools, and the resulting Ca^{2+} mobilization contributes, at least partially, to NA release in brain slices. Although *S*-nitrosothiols stimulated [^{3}H]NA release from hippocampal slices pretreated with 50 μM BAPTA-AM (a cell-permeable chelator of Ca^{2+}) in the absence of $CaCl_2$, the effect was inhibited by calmodulin antagonists [23]. Re-

cently, Yuan *et al.* [42] reported Ca^{2+}-independent interactions between calmodulin and target proteins. Further studies on the role of Ca^{2+} mobilization and calmodulin are in progress.

Another regulatory mechanism for protein(s) is ADP-ribosylation, whereby ADP-ribose is bound covalently to specific protein sites. ADP-ribosylation of tubulin inhibits polymerization for microtubule assembly [43]. $HgCl_2$ treatment *in vivo* (administration by i.p. injection) and *in vitro* (addition to brain homogenates) markedly inhibits ADP-ribosylation of tubulin, actin, and GAP-43 (a 43-kDa growth-associated protein in neurons) [44]. The addition of NEM lowers cytoskeletal F-actin levels and enhances Ca^{2+}-dependent NA release from chromaffin cells [14]. GAP-43 is thought to be involved in exocytosis from neuronal cells (for a review, see Ref. 45). Several observations suggest that GTP-binding proteins are involved in exocytosis (see the Introductions in Refs. 46 and 47), and NEM may interact with GTP-binding proteins and regulate NA release in rat hippocampal slices [16]. It has been reported that the thiol sites on actin, GAP-43, and GTP-binding proteins are targets of NO [29]. Thus, it is probable that ADP-ribosylation of these proteins may be regulated by SNC, NEM, and Hg.

Regulation of NA Release by Multiple Sulfhydryl Groups

We investigated the interactions of sulfhydryl groups in proteins regulating NA release with combinations of sulfhydryl reagents. In the absence of extracellular $CaCl_2$, the addition of 200 μM *p*-CMBA, which weakly stimulates [^{3}H]NA release alone, significantly inhibited the effect of 200 μM SNC (Table 4), and the concentration–response curve of SNC shifted to the right in the presence of $HgCl_2$ (100 μM) or *p*-CMBA (200 μM) (Fig. 1). It has been reported that radioactivity is released from [^{32}P]NAD-labeled glyceraldehyde-3-phosphate dehydrogenase in the presence of a NO donor by $HgCl_2$ *in vitro* [48] and that [^{32}P]ADP-ribosylation of the cysteine residue of the α subunit of the GTP binding protein ($G_{o\alpha}$) by pertussis toxin is inhibited by the addition of $HgCl_2$ and NO donors *in vitro* [49]. These reports and findings suggest the possibility that NO and *p*-CMBA interact with the same cysteine residues on proteins. On the other hand, the effect of 200 μM NEM on [^{3}H]NA release was enhanced synergistically by 200 μM *p*-CMBA, and the effect of 400 μM NEM was additive to the effect of *p*-CMBA (Table 4). The effect of *p*-CMBA seemed to be relatively stable, because the effect of NEM on NA release was still enhanced markedly in the slices treated with 100 μM *p*-CMBA for 5 min and then washed (see Results). The effect of SNC was additive to the effect of 200 μM NEM (Fig. 1). Thus, it is probable that the NEM-sensitive sulfhydryl groups are different from the SNC- and Hg^{2+}-sensitive groups, although further studies are needed.

T-588 is reported to stimulate NA release *in vivo* [18] and *in vitro* from rat cerebral cortical slices [20, 30]. However, T-588 inhibits NA release induced by 600 μM NEM [20]. Thus, we compared the effects of T-588 with the effects of sulfhydryl agents and SNC. The effect of T-588, including its concentration-dependence, was not modified by 200 μM *p*-CMBA (Table 4) or $HgCl_2$ (data not shown), but 1 or 3 mM T-588 did affect *p*-CMBA-treated slices (see Results). These findings suggest that the stimulatory mechanism(s) of T-588 on NA release is different from that of sulfhydryl agents. T-588 may interact only with NEM-sensitive processes.

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