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Regulation of noradrenaline release by *S*-nitroso-cysteine: inhibition in PC12 cells in a cyclic GMP-independent manner [1]

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Abstract

Nitric oxide (NO), including NO free radicals (·NO) and peroxynitrite ($OONO^-$), modulates the release of neurotransmitters from neuronal tissues. Although we reported that *S*-nitroso-cysteine stimulated noradrenaline release in brain slices, we now show that only *S*-nitroso-cysteine inhibits noradrenaline release from PC12 cells. *S*-Nitroso-cysteine inhibited, in a dose-dependent manner (up to 0.6 mM), the Ca^{2+}-dependent [^{3}H]noradrenaline release induced by ionomycin, adenosine 5′-*O*-(3-thiotriphosphate), or high K^+, from PC12 cells labeled with [^{3}H]noradrenaline. Sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine, and 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene, which specifically release NO free radicals in neutral buffer, had minimal effects on [^{3}H]noradrenaline release, although they markedly stimulated cyclic GMP accumulation. 3-Morpholinosydonimine, which releases peroxynitrite, had no effect on either [^{3}H]noradrenaline release or cyclic GMP accumulation. *S*-Nitroso-cysteine inhibited phorbol 12-myristate 13-acetate- and mastoparan (wasp venom toxin)-induced [^{3}H]noradrenaline release. These findings suggest that 1) *S*-nitroso-cysteine, but not other NO donors, inhibits some common process occurring during noradrenaline release in PC12 cells, 2) neither NO radicals, peroxynitrite, nor cyclic GMP mediate the inhibitory effects of *S*-nitroso-cysteine in PC12 cells.

Keywords: Nitric oxide (NO); *S*-Nitroso-cysteine; Noradrenaline release; cGMP; PC12 cell; Phorbol ester

1. Introduction

Nitric oxide (NO) is thought to have a wide variety of physiological functions. In the nervous system, NO has been reported to mediate synaptic plasticity such as long-term potentiation and depression, neurotoxicity, and neurotransmission. The release of neurotransmitters and neuropeptides is modulated by NO (for review, see Choi, 1993; Davis and Murphey, 1994). NO is unstable and reacts rapidly with various molecules. Molecules containing NO, such as NO free radicals, nitrosonium cations (NO^+), nitroxy anions (NO^-), higher oxides of NO, and nitrosothiols are presumed to have physiological effects, including effects on neurotransmitter release. NO has the ability to modulate the activities of proteins within functional groups, notably cofactor Fe and thiols (Vanin, 1991; Stamler et al., 1992; Stamler, 1994). For example, the activation of guanylyl cyclase by NO binding to its heme group is well known, and cyclic GMP mediates some of the effects of NO such as vasorelaxation and platelet inhibition (Murad, 1994). However, the active NO species and the mechanism underlying the actions of NO in neurotransmitter release seem to depend on the neuronal tissue type.

Previously, we reported that NO donors such as sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine stimulate noradrenaline release from the rat hippocampus in vitro (Satoh et al., 1996a,b) and in vivo (Satoh et al., 1996b) in the presence of thiol compounds, such as dithiothreitol and L-cysteine. The addition of *S*-nitroso-cysteine alone stimulated noradrenaline release (Satoh et al., 1997). The stimulatory effect of *S*-nitroso-cysteine on noradrenaline release was not mediated by cyclic GMP accumulation. These findings suggest that nitrosothiol compounds, not NO free radicals, are the active species that stimulate noradrenaline release. Thus, it is important to establish the effects of *S*-nitrosothiols on other neuronal cells.

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[1] In this paper, 'NO, nitric oxide' refers to all NO species. 'NO free radicals' indicates ·NO, which is called nitric oxide in other papers.

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The PC12 pheochromocytoma cell line is a useful model with which to study Ca^{2+}-dependent neurosecretion. Recently, we reported that Ca^{2+}-dependent noradrenaline release induced by P2 purinoceptor stimulation and high K^+ is positively regulated by dual protein kinase C and cyclic AMP dependent mechanisms (Oda et al., 1995). We have now investigated the effect of *S*-nitroso-cysteine on noradrenaline release from PC12 cells. The addition of *S*-nitroso-cysteine, but not of other chemical NO donors such as sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine, 3-morpholinosydonimine (SIN-1), or *S*-nitrosoglutathione, inhibited noradrenaline release from PC12 cells in a cyclic GMP-independent manner. The physiological roles of nitrosothiols such as *S*-nitroso-cysteine are discussed.

2. Materials and methods

2.1. Materials

L-[7,8-^{3}H]Noradrenaline (40 Ci/mmol) was purchased from Amersham. The cyclic GMP assay kit was a gift from the Yamasa-Shoyu (Chiba, Japan). Sodium nitroprusside, $NaNO_2$, $NaNO_3$ and fura-2 acetoxymethyl ester were purchased from Wako (Osaka, Japan). Adenosine 5′-*O*-(3-thiotrisphosphate) (ATPγS) and rolipram were obtained from Boehringer Mannheim (Mannheim, Germany) and the Meiji (Tokyo, Japan), respectively. Phorbol 12-myristate 13-acetate (PMA), 8-bromo cyclic GMP, mastoparan and ionomycin were purchased from Sigma (St. Louis, MO, USA). SIN-1 and *S*-nitroso-*N*-acetylpenicillamine were purchased from BIOMOL (PA, USA) and Research Biochemicals International (Natick, MA, USA), respectively. *S*-Nitroso-L-glutathione was purchased from ALEXIS (Postfach, Switzerland). 1-Hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC-18) and *S*-nitroso-cysteine were given to us by Drs. Miyazaki and Katayama in DOJINDO Laboratory (Kumamoto, Japan). 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one, an inhibitor of guanylyl cyclase, was obtained from Tocris (Britol, UK).

2.2. [^{3}H]Noradrenaline release from PC12 cells

PC12 cells (D-type, Katoh-Semba et al., 1987) were grown in Dulbecco's modified essential medium supplemented with 5% heat-inactivated fetal bovine and horse serum. At 24 h after the final change of medium, [^{3}H]noradrenaline release was measured, as described previously (Oda et al., 1995, 1996; Murayama et al., 1995). Briefly, subconfluent cells were labeled with 1 μCi/ml of L-[7,8 $-^3$H]noradrenaline (25 nM) for 2 h in a modified Tyrode's HEPES buffer (20 mM HEPES (pH 7.0), 140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$). The pH of the buffer was lowered from 7.4 to 7.0 to reduce the degradation of noradrenaline in all experiments in this study. In some experiments, 0.1 mM ascorbic acid was added to the labeling buffer (pH 7.4), or 10 μM nialamide (an inhibitor of monoamine oxidase) was added to the labeling buffer (pH 7.0). Data obtained with both procedures were similar to those obtained with the usual procedure. Detached cells were washed twice by centrifugation ($200 \times g$, 2 min) at 4°C and resuspended in Tyrode's buffer (pH 7.4). Cell suspensions (50–70 μg protein/tube) were incubated for 8 min at 37°C in Tyrode's buffer containing 0.2% fatty acid-free bovine serum albumin with or without stimulants such as ATPγS or *S*-nitroso-cysteine. The high K^+ depolarization-induced release was investigated in modified Tyrode's buffer containing 50 mM KCl and 90 mM NaCl. The reaction was terminated by the addition of 0.5 ml ice-cold Ca^{2+}- and Mg^{2+}-free Tyrode's buffer containing 5 mM EGTA and 5 mM EDTA, followed by a 30-s, $5000 \times g$ centrifugation at 4°C. The radioactivity in the supernatant was quantified with a liquid scintillation spectrometer. The data are presented as percentages of total [^{3}H]noradrenaline incorporated. This procedure had been applied to neuronal tissues including PC12 cells. The increase in tritium (over 90%) in cultured ganglion cells has been shown to be composed predominantly of intact noradrenaline and not its metabolites (Schwartz and Malik, 1993). In PC12 cells, only a small percentage (under 5%) of the [^{3}H]noradrenaline taken up by these cells in the buffer (pH 7.4) is metabolized, and the release of tritium correlates well with results of the chemical assay for noradrenaline (Scha'fer et al., 1987; Lomneth et al., 1991).

2.3. Cyclic GMP accumulation in PC12 cells

Detached PC12 cells were incubated for 5 min at 37°C with stimulants in Tyrode's buffer (pH 7.4) containing 0.5 mM isobutyl-methylxanthine and 0.2 mM rolipram. The reaction was stopped by the addition of HCl and by boiling for 2 min. Cyclic GMP levels were measured with a radioimmunoassay kit.

2.4. Measurement of $[Ca^{2+}]_i$ in PC12 cells

Intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) were estimated as described previously (Yakushi et al., 1996). Briefly, PC12 cells on dishes were incubated in 0.7 μM fura-2 acetoxymethyl ester for 20 min at 37°C in Tyrode's buffer containing 0.2% fatty acid-free bovine serum albumin. The PC12 cells were washed and detached from the dishes under a gentle stream of buffer. An aliquot of $3–5 \times 10^6$ cells was used for autofluorescence measurements at 37°C.

2.5. Statistics

The data were analyzed using a paired *t*-test and two-way analysis of variance. P values < 0.01 were considered to be significant.

3. Results

3.1. Inhibition of Ca^{2+}-dependent [^{3}H]noradrenaline release by S-nitroso-cysteine

First, we investigated the role of extracellular $CaCl_2$ in [^{3}H]noradrenaline release from prelabeled PC12 cells. The non-stimulated (basal) release was 8.1 ± 0.7% and 12.9 ± 1.3% ($n = 3$), in the absence or presence of extracellular 2 mM $CaCl_2$, respectively. The 10 μM ionomycin-stimulated [^{3}H]noradrenaline release was 9.6 ± 1.2 and 36.3 ± 1.8% ($n = 3$), in the absence or presence of 2 mM $CaCl_2$, respectively. Thus, the effect of ionomycin on noradrenaline release is completely dependent on extracellular $CaCl_2$. Fig. 1 shows the effect of *S*-nitroso-cysteine on [^{3}H]noradrenaline release in the presence of 1 mM extracellular $CaCl_2$. The addition of 0.2 mM *S*-nitroso-cysteine to the assay mixture did not significantly stimulate [^{3}H]noradrenaline release. The addition of 0.6 mM *S*-nitroso-cysteine significantly inhibited release. The addition of *S*-nitroso-cysteine markedly inhibited the ionomycin-stimulated release in a dose-dependent manner (0.2 mM–0.6 mM). The addition of *S*-nitroso-cysteine at concentrations higher than 0.6 mM strongly stimulated [^{3}H]noradrenaline release in the absence or presence of extracellular $CaCl_2$ (data not shown). We investigated the inhibitory effects of lower concentrations of *S*-nitroso-cysteine on noradrenaline release in PC12 cells. It was difficult to distinguish whether the effect of high concentrations of *S*-nitroso-cysteine was due to toxicity, although cell viability (measured by leakage of lactate dehydrogenase) did not change for at least 8 min following 1 mM *S*-nitroso-cysteine addition.

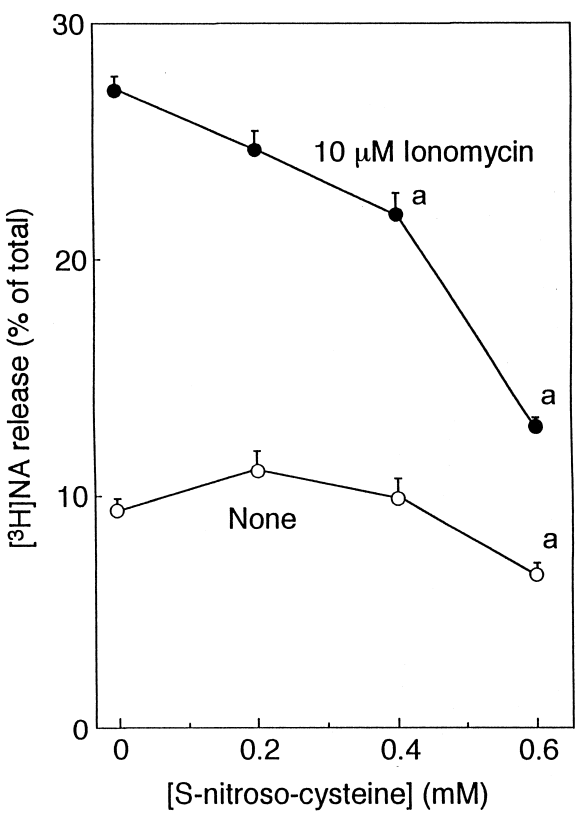

Fig. 1. Inhibition of ionomycin-stimulated [^{3}H]noradrenaline release by *S*-nitroso-cysteine. Prelabeled PC12 cells were stimulated with (●) or without (○) 10 μM ionomycin in the presence of *S*-nitroso-cysteine at the concentrations indicated. Extracellular $CaCl_2$ concentration was 1.0 mM. NA, noradrenaline. Points are means ± S.E.M. of three or four independent experiments performed in triplicate. Statistical significance was determined by two-way analysis of variance, $^aP < 0.01$, vs. values without SNC.

Table 1
Effects of NO donors on [^{3}H]noradrenaline release from PC12 cells

Addition	[^{3}H]Noradrenaline release (% of total)	
	None	10 μM Ionomycin
None	9.6 ± 0.6	26.5 ± 1.3
SNC	6.3 ± 0.3	12.2 ± 0.8
SNP	12.3 ± 0.7	30.1 ± 0.2
SNAP	9.8 ± 2.0	28.9 ± 1.1
NOC-18	9.6 ± 1.6	29.0 ± 2.5
SIN-1	12.5 ± 0.9	28.7 ± 1.0
$NaNO_2$	8.8 ± 1.0	26.7 ± 2.2
$NaNO_3$	9.5 ± 0.4	24.4 ± 1.1
S-Nitrosoglutathione	9.0 ± 1.0	25.0 ± 1.2

Prelabeled PC12 cells were stimulated with or without 10 μM ionomycin in the presence of various NO donors at concentrations of 0.6 mM.
SNC, *S*-nitroso-cysteine.
SNP, sodium nitroprusside.
SNAP, *S*-nitroso-*N*-acetylpenicillamine.
NOC-18, 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene.
SIN-1,3-morpholinosydonimine.
Values are means ± S.D. of three determinations from a typical experiment. Data are representative of three independent experiments.

Table 1 shows the effects of 0.6 mM of various NO donors on ionomycin-stimulated [^{3}H]noradrenaline release from PC12 cells. Sodium nitroprusside increased [^{3}H]noradrenaline release and enhanced the ionomycin-stimulated reaction. However, the effect of sodium nitroprusside was slight and not significant in two other experiments. NOC-18, which specifically releases NO free radicals in neutral buffers containing no co-factor, such as a phosphate buffer (pH 7.4) (Hrabie et al., 1993), had no effect on [^{3}H]noradrenaline release. SIN-1, which yields peroxynitrite (Hogg et al., 1992), had no effect. Although 0.6 mM SIN-1 seemed to increase [^{3}H]noradrenaline release slightly (Table 1), SIN-1 had no stimulatory effect in two other experiments. Neither *S*-nitroso-*N*-acetylpenicillamine, $NaNO_2$, nor $NaNO_3$ affected the basal or ionomycin-stimulated [^{3}H]noradrenaline release. *S*-Nitrosoglutathione had no effect on basal or ionomycin-stimulated [^{3}H]noradrenaline release. The data were reproducible, and none of the NO donors tested, except *S*-nitroso-cysteine, had an inhibitory effect at low (0.2 and 0.4 mM) and high (1 mM) concentrations. Previously we reported that the combination of sodium nitroprusside or *S*-nitroso-*N*-acetylpenicil-

lamine and dithiothreitol or cysteine yielded *S*-nitrosothiols and stimulated noradrenaline release from brain slices (Satoh et al., 1996a,b). However, no NO donors used, except *S*-nitroso-cysteine, had an effect in the presence of dithiothreitol (5 mM), cysteine (1 mM), or glutathione (1 mM).

Stimulation of P2 purinoceptors and addition of high K^+ increased Ca^{2+} influx and catecholamine release (Oda et al., 1995, 1996). ATPγS or KCl-stimulated [^{3}H]noradrenaline release was inhibited by *S*-nitroso-cysteine in a similar and dose-dependent manner. [^{3}H]Noradrenaline release in the presence of 0.6 mM *S*-nitroso-cysteine was decreased to 20–30% from its control levels (Table 2). Previously we reported that PMA stimulated [^{3}H]noradrenaline release from PC12 cells via activation of protein kinase C in the presence of $CaCl_2$ (Oda et al., 1995). In the present study, stimulation with 100 nM PMA for 8 min increased [^{3}H]noradrenaline release markedly; the basal release and the PMA-stimulated release were 7.5 ± 0.4 and $17.6 \pm 1.3\%$ ($n = 3$), respectively. The addition of 0.6 mM *S*-nitroso-cysteine reduced by $47.6 \pm 4.1\%$ ($n = 3$) the net increase induced by 100 nM PMA.

3.2. Inhibition of Ca^{2+}-independent [^{3}H]noradrenaline release by S-nitroso-cysteine

Mastoparan, a wasp venom toxin, increases the secretion of neurotransmitters and hormones from a variety of cell types, including PC12 cells (Murayama et al., 1996). The addition of mastoparan stimulated [^{3}H]noradrenaline release from PC12 cells in the presence or absence of extracellular $CaCl_2$ (Table 3). The effect of 10 μM mastoparan in the presence of $CaCl_2$ was less than that in the absence of $CaCl_2$, as described previously (Murayama et al., 1996). The maximal [^{3}H]noradrenaline release (41.2 ± 0.6, percent of total, $n = 3$) was obtained with 20 μM mastoparan in the absence of 2 mM $CaCl_2$. The effects of mastoparan are not dependent on the activation of protein kinase C, as the [^{3}H]noradrenaline release induced by mastoparan in the presence of 3 μM calphostin C, which greatly inhibits the effect of PMA (Oda et al., 1995), was identical to the release induced in the absence of the inhibitor. Additionally, mastoparan was effective in protein kinase C-depleted PC12 cells, which had been prepared by incubation with 1 μM PMA for 24 h, as previously reported (Oda et al., 1995). *S*-Nitroso-cysteine dose dependently inhibited the effects of mastoparan in either the presence or the absence of $CaCl_2$. The mastoparan-stimulated [^{3}H]noradrenaline release was significantly decreased, by 41 ± 5 and $42 \pm 6\%$, on addition of 0.4 mM *S*-nitroso-cysteine ($n = 3$) in the absence or presence of extracellular $CaCl_2$, respectively.

Table 2
Inhibition of ATPγS- and KCl-induced [^{3}H]noradrenaline release by *S*-nitroso-cysteine

Addition	Net increase in [^{3}H]noradrenaline release (%)		
	None	0.3 mM SNC	0.6 mM SNC
300 μM ATPγS	100	64, 54	20.9 ± 3.9[a]
50 mM KCl	100	72, 60	32.8 ± 4.9[a]

Prelabeled PC12 cells were stimulated with 300 μM ATPγS or 50 mM KCl in the presence or absence of 0.6 mM *S*-nitroso-cysteine (SNC). Data were calculated as percentages of net increase in [^{3}H]noradrenaline release caused by the stimulants. The data are presented as means ± S.E.M. of three independent experiments performed in triplicate. The data for 0.3 mM *S*-nitroso-cysteine were from two independent experiments. The basal, ATPγS- and KCl-stimulated [^{3}H]noradrenaline releases were 5.0 ± 2.1, 17.2 ± 2.2 and $17.0 \pm 1.3\%$, respectively. The basal [^{3}H]noradrenaline release in the presence of 0.6 mM *S*-nitroso-cysteine was $3.7 \pm 1.5\%$. Statistical significance was determined by paired *t*-test and by two-way analysis of variance.
[a] $P < 0.01$, vs. none.

Table 3
Inhibition of mastoparan-stimulated [^{3}H]noradrenaline release by *S*-nitroso-cysteine

Addition	[^{3}H]Noradrenaline release (% of total)	
	0.2 mM EGTA	2 mM $CaCl_2$ and 0.2 mM EGTA
None	2.9 ± 2.8	8.4 ± 2.3
+0.2 mM SNC	2.8 ± 2.5	9.0 ± 3.1
+0.4 mM SNC	2.3 ± 2.2	5.8 ± 2.2
10 μM Mastoparan	42.9 ± 0.4	29.4 ± 0.4
+0.2 mM SNC	36.3 ± 1.1	22.3 ± 1.2
+0.4 mM SNC	18.7 ± 2.1	14.6 ± 2.3

Prelabeled PC12 cells were stimulated with or without 10 μM mastoparan in the presence or absence of 2 mM $CaCl_2$ EGTA (0.2 mM) and the indicated concentrations of *S*-nitroso-cysteine (SNC) were subsequently added to the assay mixture. Data are presented as means ± S.D. of three determinations in a typical experiment. Data are representative of three independent experiments.

3.3. Increase of $[Ca^{2+}]_i$ by S-nitroso-cysteine in PC12 cells

We investigated whether the inhibitory effect of *S*-nitroso-cysteine on [^{3}H]noradrenaline release was due to inhibition of $[Ca^{2+}]_i$ increase. The addition of *S*-nitroso-cysteine produced a continuous, dose-dependent $[Ca^{2+}]_i$ increase from an intracellular Ca^{2+} pool in PC12 cells [2]. The increase in $[Ca^{2+}]_i$ caused by 0.6 mM *S*-nitroso-cysteine for 350 s in the presence or absence of extracellular $CaCl_2$ (1 mM) was 290 ± 15 and 310 ± 40 nM ($n = 3$), respectively. Additionally, *S*-nitroso-cysteine did not inhibit the increase in $[Ca^{2+}]_i$ caused by 10 μM ionomycin (data not shown). These findings suggest that the inhibitory effect of *S*-nitroso-cysteine on [^{3}H]noradrenaline release is not due to $[Ca^{2+}]_i$ mobilization.

[2] Naganuma, T., Murayama, T. and Nomura, Y., manuscript in preparation.

3.4. Effects of NO donors on cyclic GMP accumulation

It has been shown that PC12 cells treated with sodium nitroprusside accumulate cyclic GMP and have increased tyrosine hydroxylase activity (Roskoski and Roskoski, 1987). To test for the potential involvement of cyclic GMP in the regulation of noradrenaline release, PC12 cells were treated with various NO donors. The addition of sodium nitroprusside stimulated cyclic GMP accumulation in a dose-dependent manner, reaching a maximum 300 μM (Fig. 2). Biphasic (stimulatory and inhibitory) effects of *S*-nitroso-cysteine on cyclic GMP were observed when PC12 cells were incubated with increasing concentrations. *S*-Nitroso-cysteine markedly stimulated cyclic GMP accumulation at concentrations from 3 to 30 μM. However, *S*-nitroso-cysteine over 30 μM inhibited the accumulation of cyclic GMP, while the cyclic GMP level after stimulation with 300 μM *S*-nitroso-cysteine was 10 times higher than the basal level. The addition of *S*-nitroso-*N*-acetylpenicillamine, $NaNO_2$, or NOC-18, but not $NaNO_3$ or SIN-1, markedly stimulated cyclic GMP in a dose-dependent manner, without any inhibitory phase; 25.5 ± 3.3 by 300 μM *S*-nitroso-*N*-acetylpenicillamine, 10.5 ± 2.0 by 500 μM $NaNO_2$ and 15.2 ± 2.2 pmol/10^6 cells by 500 μM NOC-18 (mean ± S.D. of three determinations in a typical experiment, similar data were obtained in another experiment).

Oxyhemoglobin and carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide), which are NO free radical scavengers, effectively inhibit cyclic GMP accumulation by NO donors in the hippocampus (Satoh et al., 1996b). These scavengers also inhibit cyclic GMP accumulation by NO donors including *S*-nitroso-cysteine cysteine in PC12 cells. The increase in cyclic GMP caused by *S*-nitroso-*N*-acetylpenicillamine in PC12 cells was not inhibited by the scavengers, as had been demonstrated for the hippocampus (Satoh et al., 1996b). The NO radical scavengers did not modify the 10 μM ionomycin-stimulated [^{3}H]noradrenaline release; 27.3 ± 1.2 and $28.4 \pm 1.5\%$ ($n = 3$), in the presence of 500 μg/ml oxyhemoglobin and 1 mM carboxy-PTIO, respectively, which is almost the same as without the scavengers. Furthermore, the ionomycin-stimulated [^{3}H]noradrenaline release was inhibited by 0.6 mM *S*-nitroso-cysteine in the presence of scavengers; 12.5 ± 1.2 and $14.0 \pm 1.3\%$ ($n = 3$), in the presence of oxyhemoglobin and carboxy-PTIO, respectively, which is almost the same as without the scavengers. Also, the addition of 0.1 mM 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, a selective inhibitor of NO-sensitive guanylyl cyclase, did not modify the inhibitory effect of *S*-nitroso-cysteine, and addition of 1 and 2 mM 8-bromo cyclic GMP in the assay mixture did not inhibit [^{3}H]noradrenaline release. These findings eliminate the possibility of cyclic GMP and NO free radical involvement in the regulation of noradrenaline release by *S*-nitroso-cysteine.

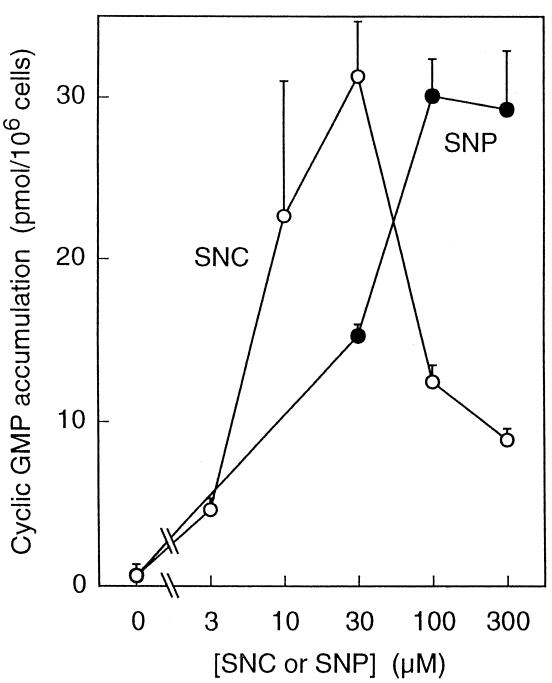

Fig. 2. Effects of NO donors on cyclic GMP accumulation in PC12 cells. PC12 cells were incubated with the indicated concentrations of *S*-nitroso-cysteine (SNC, ○) and sodium nitroprusside (SNP, ●). Results indicate means ± S.D. of three determinations in a typical experiment. Data are representative of two independent experiments.

4. Discussion

The release of neurotransmitters and neuropeptides is thought to be both positively and negatively regulated by NO. Various NO donors have been shown to stimulate, but not inhibit, noradrenaline release from hippocampal slices (Lonart et al., 1992; Lonart and Johnson, 1995) and synaptosomes (Meffert et al., 1994). We also have reported that sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine stimulate noradrenaline release in the presence of dithiothreitol or L-cysteine (Satoh et al., 1996a,b), and that *S*-nitroso-cysteine itself stimulates noradrenaline release (Satoh et al., 1997) from rat hippocampal slices. The addition of *S*-nitroso-*N*-acetylpenicillamine and L-cysteine (Satoh et al., 1996b), or 0.5 mM *S*-nitroso-cysteine (Satoh et al., 1997) stimulates endogenous noradrenaline release within 30 min in vivo (microdialysis assay). Myers et al. (1990) showed that the endothelium-derived relaxing factor is more likely to be *S*-nitroso-cysteine than NO free radicals. Recently, Gow et al. (1997) reported that NO free radicals react with thiols to form *S*-nitrosothiol under physiological conditions. These findings suggest that *S*-nitroso-cysteine is one of the physiological NO molecules. In the present study, we investigated the roles of *S*-nitroso-cysteine and other NO donors in noradrenaline release from PC12 cells. We found that (1) only *S*-nitroso-cysteine is effective, (2) *S*-nitroso-cysteine inhibits,

but does not stimulate, [^{3}H]noradrenaline release from PC12 cells. These findings provide evidence that *S*-nitroso-cysteine, not NO free radicals, plays an inhibitory role in noradrenaline release from PC12 cells.

In general, NO molecules are labile in physiological solutions and react rapidly with various substances including molecular oxygen, superoxide anions, hemes and thiols (Vanin, 1991; Stamler et al., 1992; Stamler, 1994). Thus, we investigated the pharmacological profile of NO molecules involved in the inhibition of noradrenaline release, using various NO donors. Sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine, and $NaNO_2$, which are non-selective NO donors, stimulated cyclic GMP accumulation in PC12 cells (Fig. 2), but had no effect on [^{3}H]noradrenaline release (Table 1). Neither SIN-1 nor NOC-18, which predominantly release peroxynitrite and NO free radicals, respectively, modified [^{3}H]noradrenaline release. Only *S*-nitroso-cysteine inhibited in a dose-dependent manner the [^{3}H]noradrenaline release induced by stimulants (Fig. 1). Low concentrations of *S*-nitroso-cysteine (up to 30 μM) markedly stimulated cyclic GMP accumulation in PC12 cells (Fig. 2), although *S*-nitroso-cysteine had no inhibitory effects on noradrenaline release at low concentrations (Fig. 1). The addition of 8-bromo cyclic GMP did not inhibit basal or ionomycin-stimulated [^{3}H]noradrenaline release. These findings suggest that NO free radicals, peroxynitrite, or cyclic GMP do not mediate the inhibitory effects of *S*-nitroso-cysteine on PC12 cells. Previously, we reported that *S*-nitroso-cysteine is incorporated into rat hippocampal slices via an L-type-like amino acid transporter (Satoh et al., 1997). This suggests that only *S*-nitroso-cysteine, not NO free radicals, is selectively incorporated and inhibits [^{3}H]noradrenaline release in PC12 cells. Our findings concur with the hypothesis that NO forms nitrosyl complexes with thiol ligands, including cysteine and proteins, and that these complexes are stable and potent bioactive molecules in physiological systems (Myers et al., 1990; Vanin, 1991).

It has been proposed that *S*-nitroso-cysteine induces *S*-nitrosylation of the *N*-methyl-D-aspartate receptor–channel complex (Lei et al., 1992; Lipton et al., 1993). In PC12 cells, stimulation of P2 purinoceptors and their subsequent depolarization caused by high K^+, increase Ca^{2+} influx via both voltage-insensitive and -sensitive Ca^{2+} channels, and stimulate the release of catecholamines (Oda et al., 1995, 1996). Addition of *S*-nitroso-cysteine inhibits ATP- and high-K^+-stimulated [^{3}H]noradrenaline release (Table 2). The inhibitory effect of *S*-nitroso-cysteine, however, is not derived from the inhibition of Ca^{2+} channels and $[Ca^{2+}]_i$ mobilization in PC12 cells, because; (1) *S*-nitroso-cysteine inhibits not only ATP receptor-mediated and high-K^+-stimulated but also Ca^{2+} ionophore-stimulated noradrenaline release (Fig. 1 and Table 2), (2) *S*-nitroso-cysteine also inhibits the effect of PMA, a protein kinase C activator. PMA does not stimulate Ca^{2+} influx into PC12 cells (Fasolato et al., 1990), although the stimulatory effect of PMA is dependent on $CaCl_2$ (Oda et al., 1995), (3) *S*-nitroso-cysteine inhibits the effects of mastoparan, which stimulates [^{3}H]noradrenaline release in the absence of $CaCl_2$ (Table 3), (4) *S*-nitroso-cysteine alone, at concentrations of up to 0.6 mM, produces an increase in $[Ca^{2+}]_i$ in PC12 cells.

It has been shown that *S*-nitroso-cysteine inhibits protein kinase C activity, and that this inactivation is reversed by the addition of dithiothreitol to B16 melanoma cells (Gopalakrishna et al., 1993). The [^{3}H]noradrenaline release stimulated by ATP, high-K^+ or PMA is inhibited by protein kinase C inhibitors such as calphostin C, and in protein kinase C-deficient PC12 cells (Oda et al., 1995). The addition of *S*-nitroso-cysteine inhibits the effect of both Ca^{2+} influx stimulators such as ATP and ionomycin, and PMA. However, *S*-nitroso-cysteine also inhibits the effect of mastoparan, which stimulates noradrenaline release by a protein kinase C-independent pathway.

NO donors including *S*-nitroso-cysteine regulate the activity of many other proteins by covalent modification such as *S*-nitrosylation, NAD linkage, or ADP-ribosylation, and these modifications are relatively stable for long periods (Williams et al., 1992; McDonald and Moss, 1993; Asahi et al., 1995). Under our experimental conditions, the inhibitory effect of *S*-nitroso-cysteine was not washed out, as the effects of ATP and ionomycin on [^{3}H]noradrenaline release in PC12 cells treated with 0.6 mM *S*-nitroso-cysteine for 5 min and then washed, decreased by 30–40% compared with those in non-treated cells (data not shown). Thus, we presume that *S*-nitroso-cysteine induces the covalent modification of proteins other than Ca^{2+} channels or protein kinase C, which are involved in noradrenaline release in PC12 cells. It is reported that several proteins, such as small GTP-binding proteins (Rab family), synapsin, and synaptophysin, regulate exocytosis and synaptic vesicle trafficking (for review, see Bennett and Scheller, 1994). It has been demonstrated that NO stimulates guanine nucleotide exchange on small GTP-binding protein (Lander et al., 1995). It is probable that *S*-nitroso-cysteine has some inhibitory effect(s), which overcome the increase in $[Ca^{2+}]_i$ by *S*-nitroso-cysteine, on noradrenaline release in PC12 cells.

As mentioned above, NO donors stimulate noradrenaline release (Lonart et al., 1992; Meffert et al., 1994; Lonart and Johnson, 1995). Co-addition of NO donors and L-cysteine (Satoh et al., 1996a,b) and *S*-nitroso-cysteine (Satoh et al., 1997) also stimulated noradrenaline release from rat hippocampus in vitro and in vivo. In PC12 cells, however, *S*-nitroso-cysteine inhibits noradrenaline release. In differentiated PC12 cells, with an acquired neuron-like phenotype from prolonged treatment with nerve growth factor (50 ng/ml, 48 h), *S*-nitroso-cysteine inhibits noradrenaline release (data not shown). Thus, there is a difference between the effect of *S*-nitroso-cysteine in PC12 cells and on brain preparations that requires further study. The metabolism of *S*-nitroso-cysteine in cultured cells

such as PC12 cells may be different from that in rat hippocampus. To find the target protein(s) of *S*-nitroso-cysteine or the mechanism by which *S*-nitroso-cysteine affects PC12 cells, it is important to characterize the differences in noradrenaline release, the inhibitory effects of NO in PC12 cells, and its stimulatory effects in other tissues.

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