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Regulation of cyclic GMP and cyclic AMP production by *S*-nitroso-cysteine in rat thymocytes

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Abstract

Nitrogen monoxides regulate cellular functions via cyclic GMP accumulation induced by nitric oxide (NO). However, the effects of NO on the cyclic AMP system have not been studied in detail. In this study, we investigated the effects of various NO donors on cyclic GMP and cyclic AMP accumulation in rat thymocytes. Addition of *S*-nitroso-cysteine stimulated cyclic GMP accumulation at concentrations up to 10 μM, but was inhibitory at higher concentrations. Other NO donors such as sodium nitroprusside stimulated cyclic GMP accumulation markedly without causing inhibition. *S*-Nitroso-cysteine, but not other NO donors, inhibited forskolin-stimulated cyclic AMP accumulation in intact thymocytes and thymocyte membrane preparations. The inhibitory effect of *S*-nitroso-cysteine on cyclic AMP accumulation in membranes was partially reversed by dithiothreitol treatment. These findings suggest that the cyclic AMP system in thymocytes is specifically modified by *S*-nitroso-cysteine, and not by the NO/cyclic GMP system.

Keywords: Nitrogen monoxide; *S*-Nitroso-cysteine; Cyclic nucleotide; Adenylyl cyclase; Thymocyte

1. Introduction

The thymus is a key organ of the immune system and is the primary site of T cell lymphopoiesis. The presence of α_2-adrenoceptors (Marchetti et al., 1994) and adenosine receptors (Szondy, 1994) in the rat thymus has been reported, as well as cyclic AMP potentiated DNA fragmentation and thymocyte death (McConkey et al., 1993; Szondy, 1994). Additionally, the presence of muscarinic acetylcholine receptors, elevation of cyclic GMP levels, and regulation of functions by receptor stimulation have been reported (Maslinski et al., 1988). Previously, we reported on the presence of muscarinic M_3 receptors and carbachol-stimulated DNA fragmentation (Yamada et al., 1997), and showed that the addition of chemical nitric oxide (NO) donors such as sodium nitroprusside and dibutyryl cyclic GMP inhibited DNA synthesis in rat thymocytes (Yamada et al., 1998). These reports suggest that cyclic AMP and cyclic GMP act as second messengers in thymocytes.

Nitrogen monoxides including NO are important cellular regulators in various systems including the nervous and immune systems. However, the broader chemistry of nitrogen monoxides involves various species such as NO, the nitrosonium cation (NO^+) and the nitroxyl anion (NO^-) (for review, see Stamler et al., 1992). One of the well-established effects of NO is the activation of guanylyl cyclase/cyclic GMP production. The functions of this pathway have been reviewed by Murad (1994). Although multiple nitric oxide synthase systems exist in the adult rat thymus (Downing, 1994), the levels of cyclic GMP in thymocytes have not been established. The effects of NO (and/or cyclic GMP) on the other second messenger, cyclic AMP, in thymocytes have also not been studied. In this study, we investigated the effects of chemical NO donors on cyclic GMP and cyclic AMP levels in rat thymocytes to examine the interactions between NO and cyclic AMP levels. All NO donors including *S*-nitroso-cysteine used in this study stimulated cyclic GMP accumulation via NO. High concentrations of *S*-nitroso-cysteine, but not of other NO donors, inhibited cyclic GMP and cyclic AMP in rat thymocytes. The inhibitory effect of *S*-nitroso-cysteine on adenylyl cyclase in the membranes was

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relatively stable, but treatment with dithiothreitol, a reducing agent, blocked this inhibition. The physiological role of *S*-nitroso-cysteine as an intermediate of nitrogen monoxides is discussed.

2. Materials and methods

2.1. Animals and materials

Male Sprague–Dawley rats (5 weeks) were obtained from NRC Haruna (Gunma, Japan). They were provided with a commercial feed ad libitum for 1 week under specific pathogen-free conditions before use. All animal experiments were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the animal care and use committee of Hokkaido University. Cyclic GMP and cyclic AMP assay kits were donated by the Yamasa-Shoyu (Chiba, Japan). *S*-Nitroso-*N*-acetylpenicillamine, sodium nitroprusside and $NaNO_2$ were obtained from Wako Pure Chemical (Osaka, Japan). *S*-Nitroso-cysteine and 1-hydroxy-2-*oxo*-3,3-bis(2-aminoethyl)-1-triazene (NOC-18) were gifts from Drs. Katayama and Miyazaki (Dojindo Lab., Kumamoto, Japan); *S*-nitroso-cysteine was synthesized as described by Lei et al. (1992) with modifications. Guanosine 5′-(3-*O*-thio)triphosphate (GTPγS) was purchased from Boehringer-Mannheim (Germany).

2.2. Isolation of thymocytes

Rats were killed by decapitation and their thymuses were removed. We used decapitation instead of deep anesthesia in order to avoid the effects of anesthetics and the contamination of the thymocyte preparation with blood. Stem cell suspensions were obtained by teasing the tissue through a nylon mesh filter (300×300 μm) in modified Krebs–Ringer buffer (119 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM glucose, 10 mM HEPES, pH 7.2), as described previously (Yamada et al., 1997, 1998). The cells were resuspended in ammonium chloride–Tris buffer (144 mM NH_4Cl, 17 mM Tris, pH 7.2) and incubated for 5 min at 4°C in order to hemolyse the erythrocytes and other cells. The cells were washed three times by centrifugation ($300 \times g$, 2 min, 4°C) and resuspended in buffer. The contamination of antigen-presenting cells such as dendritic cells and macrophages was less than 2%, as measured by morphological observation and by reactivity with anti-rat macrophage antibodies (Inter-cell Tec.). The purity of the thymocytes was higher than 95%.

2.3. Measurement of cyclic GMP and cyclic AMP content

The thymocytes ($(2–4) \times 10^5$ cells/tube) were incubated with the indicated NO donors for 5 min at 37°C in Krebs–Ringer buffer supplemented with 0.2 mM Ro-20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidione), 0.2 mM rolipram and 0.5 mM 3-isobutyl-1-methylxanthine as phosphodiesterase inhibitors. Incubations were terminated by acidification with HCl to 0.2 M and then boiling for 1 min. The cyclic GMP and cyclic AMP present in the supernatant were quantified by radioimmunoassay.

2.4. Measurement of adenylyl cyclase activity

The thymocytes were homogenized with 10 volumes of 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine, and 4 μg ml^{-1} of DNase using a glass-Teflon homogenizer. The homogenate was centrifuged at $300 \times g$ for 1 min, and the supernatant was further centrifuged at $40\,000 \times g$ for 20 min at 4°C. The pellets were resuspended in 20 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$ and 0.2 mM EGTA, and washed twice with the same buffer. The assay for adenylyl cyclase activity was initiated by the addition of membrane suspensions to 200 μl of 20 mM Tris–HCl (pH 7.4) containing 5 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM ATP, 5 mM phosphocreatine, 30 unit ml^{-1} of phosphocreatine kinase, three different phosphodiesterase inhibitors, and 0.1% bovine serum albumin. Further additions were as indicated in the legends of the figures and tables. After 20 min at 30°C, the reaction was stopped by acidification with HCl and boiling. Protein was determined using a BioRad assay kit with bovine serum albumin as the standard.

2.5. Statistical significances

Data were analyzed using the unpaired *t*-test. P values < 0.01 were considered to be significant.

3. Results

3.1. NO-dependent stimulatory effect and -independent inhibitory effect of S-nitroso-cysteine on cyclic GMP accumulation in rat thymocytes

Fig. 1 shows that the addition of *S*-nitroso-cysteine at concentrations up to 10 μM remarkably stimulated cyclic GMP accumulation. However, *S*-nitroso-cysteine at concentrations over 10 μM inhibited cyclic GMP accumulation. The ED_{50} of *S*-nitroso-cysteine in the stimulatory phase was 3.6 ± 2.2 μM ($n = 3$), and the ED_{50} in the inhibitory phase was 140 ± 60 μM ($n = 3$). Thus, *S*-nitroso-cysteine had a biphasic effect on cyclic GMP accumulation in rat thymocytes. The addition of sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine stimulated cyclic GMP accumulation in a concentration-dependent manner, without an inhibitory phase (Fig. 2). NOC-18 also stimulated cyclic GMP accumulation in a concentration-dependent up to 500 μM (see Table 1) without caus-

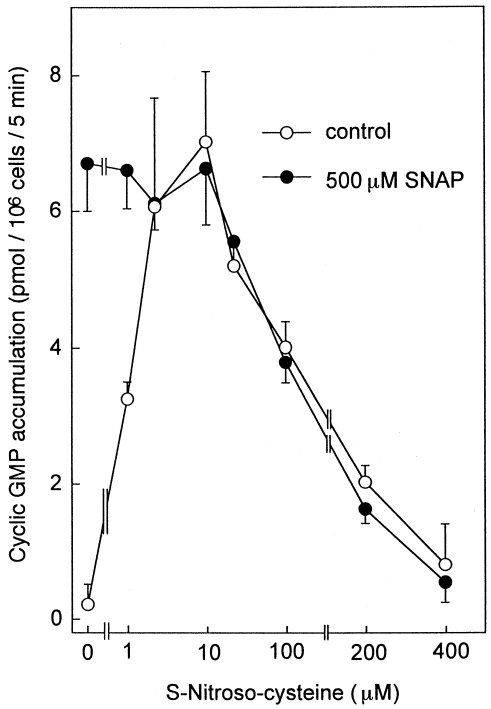

Fig. 1. Stimulatory and inhibitory effects of *S*-nitroso-cysteine on cyclic GMP accumulation in rat thymocytes. Rat thymocytes were incubated with the indicated concentrations of *S*-nitroso-cysteine in the presence (●) and absence (○) of 500 μM *S*-nitroso-*N*-acetylpenicillamine. SNAP, *S*-nitroso-*N*-acetylpenicillamine. Data are means ± S.E. of three independent experiments.

ing an inhibitory phase. The addition of 500 μM $NaNO_2$, but not $NaNO_3$, stimulated cyclic GMP accumulation. The increase in cyclic GMP induced by NO donors including *S*-nitroso-cysteine seemed to be derived from the release of NO, because the addition of 1 mg/ml of oxyhemoglobin, which binds and inactivates NO (Rettori et al., 1993; Lonart and Johnson, 1995; Satoh et al., 1996a,b), abolished the effects of *S*-nitroso-cysteine, sodium nitroprusside, and NOC-18 (Table 1). These findings show that the effect of *S*-nitroso-cysteine on cyclic GMP accumulation is exerted via NO. Penicillamine and *N*-acetylpenicillamine (400 μM), the degradation products of *S*-nitroso-*N*-acetylpenicillamine, and cysteine (400 μM) had no effect on cyclic GMP accumulation (data not shown). *S*-Nitrosoglutathione (400 μM) did not stimulate cyclic GMP accumulation. *S*-Nitroso-*N*-acetylpenicillamine-stimulated increases in cyclic GMP were not inhibited by oxyhemoglobin.

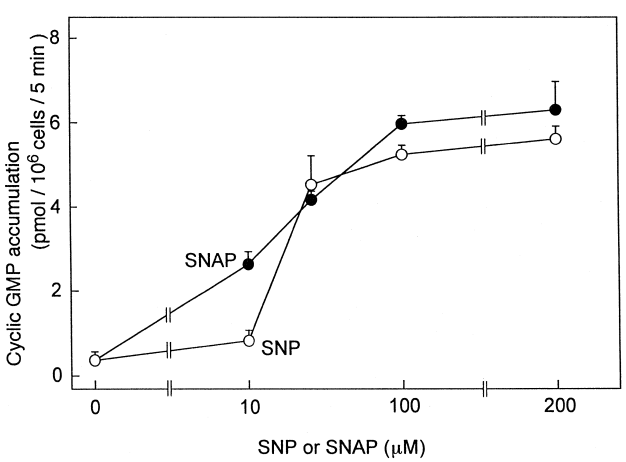

Fig. 2. Stimulatory effects of sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine on cyclic GMP accumulation. Rat thymocytes were incubated with the indicated concentrations of sodium nitroprusside (○) and *S*-nitroso-*N*-acetylpenicillamine (●). SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine. Data are means ± S.E. of three independent experiments.

Table 1
Inhibitory effect of oxyhemoglobin on NO donor-stimulated cyclic GMP accumulation in rat thymocytes

Addition	Cyclic GMP accumulation (pmol/10^6 cells per 5 min)	
	None	Oxyhemoglobin (1 mg ml^{-1})
None	0.07 ± 0.01	0.08 ± 0.01
SNC (10 μM)	10.9 ± 1.5	0.13 ± 0.02
SNP (500 μM)	12.1 ± 1.5	0.12 ± 0.01
SNAP (500 μM)	11.5 ± 1.8	16.3 ± 1.6
NOC-18 (500 μM)	3.40 ± 0.41	0.10 ± 0.12
$NaNO_2$ (500 μM)	3.05 ± 0.22	Not determined

The thymocytes were incubated with the indicated NO donors in the presence or absence of 1 mg/ml of oxyhemoglobin. SNC, *S*-nitroso-cysteine. SNP, sodium nitroprusside. SNAP, *S*-nitroso-*N*-acetylpenicillamine. Data shown are means ± S.D. of three determinations in a typical experiment and are representative of three independent experiments. The absolute values of three independent experiments agreed to within ± 30%.

The inhibitory effect of *S*-nitroso-cysteine at higher concentrations was observed in the presence of 500 μM *S*-nitroso-*N*-acetylpenicillamine (Fig. 1). Under these conditions, *S*-nitroso-cysteine did not further enhance the *S*-nitroso-*N*-acetylpenicillamine-stimulated accumulation of cyclic GMP. The inhibitory effects of *S*-nitroso-cysteine at high concentrations in the presence of *S*-nitroso-*N*-acetylpenicillamine were observed in the presence of 1 mg/ml of oxyhemoglobin.

3.2. Inhibitory effect of S-nitroso-cysteine on cyclic AMP accumulation in rat thymocytes

Next, we investigated the effects of NO donors on cyclic AMP accumulation in rat thymocytes. The addition of 400 μM *S*-nitroso-cysteine, but not sodium nitroprusside or *S*-nitroso-*N*-acetylpenicillamine, remarkably inhibited the basal (non-stimulated) cyclic AMP content (Table 2). Also *S*-nitroso-cysteine inhibited forskolin-stimulated cyclic AMP accumulation in intact thymocytes (Table 2 and Fig. 3). Forskolin-stimulated cyclic AMP accumulation was inhibited by the addition of *S*-nitroso-cysteine to the assay mixture in a concentration-dependent manner (Fig. 3). Significant inhibition was obtained with 100 μM

Table 2
Effects of NO donors on cyclic AMP accumulation in rat thymocytes

Addition	Cyclic AMP accumulation (pmol/10^6 cells per 5 min)	
	None	10 μM Forskolin
None	2.1 ± 0.2	21.1 ± 1.3
400 μM SNC	0.8 ± 0.2	2.8 ± 1.9
400 μM SNP	2.4 ± 0.1	21.4 ± 1.3
400 μM SNAP	2.5 ± 0.1	24.9 ± 1.6

The thymocytes were incubated with the indicated NO donors in the presence or absence of 10 μM forskolin. SNC, *S*-nitroso-cysteine. SNP, sodium nitroprusside. SNAP, *S*-nitroso-*N*-acetylpenicillamine. Data shown are means ± S.D. of three determinations in a typical experiment and are representative of three independent experiments. The absolute values of three independent experiments agreed to within ± 30%.

S-nitroso-cysteine and the ED_{50} values for inhibition, in the range of concentrations examined, was 250 ± 30 μM ($n = 3$).

It has been reported that NO donors increase cytosolic free Ca^{2+} concentrations in PC12 cells (Clementi et al., 1996). Some types of adenylyl cyclase are regulated by Ca^{2+} (for review, see Tang and Gilman, 1992) and a rise in cytosolic free Ca^{2+} concentrations induced by Ca^{2+} ionophores has been shown to inhibit cyclic AMP accumulation in 3T3 cells (Murayama and Ui, 1985). In our study with rat thymocytes, the addition of 10 μM ionomycin inhibited 10 μM-forskolin-stimulated cyclic AMP accumulation (5.1 ± 1.0 and 21.1 ± 1.3 pmol cyclic AMP/10^6 cells per 5 min ($n = 3$) in the presence or absence of 10 μM ionomycin, respectively). However, basal and forskolin-stimulated cyclic AMP accumulation was not modified by the removal of extracellular $CaCl_2$ or by pretreatment for 10 min with 50 μM BAPTA-AM, a cell-permeable chelator of Ca^{2+}, in the absence of $CaCl_2$. These reactions were not modified by pretreatment with the calmodulin antagonist, 10 μM W-7 (data not shown). Also the inhibitory effects of 500 μM *S*-nitroso-cysteine on basal and forskolin-stimulated cyclic AMP accumulation were not modified by either treatment.

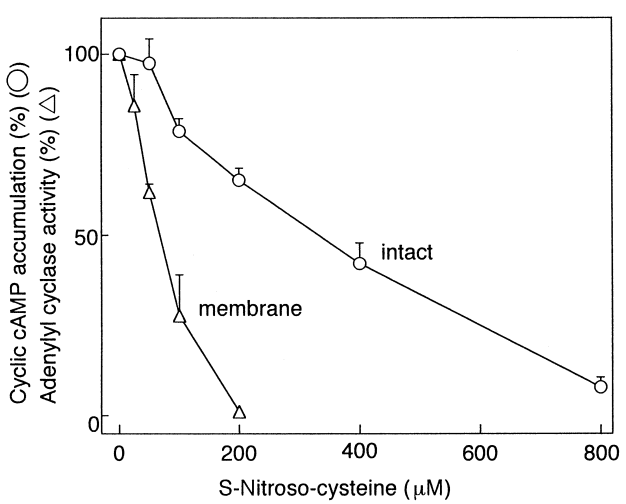

Fig. 3. Inhibitory effect of *S*-nitroso-cysteine on cyclic AMP accumulation and adenylyl cyclase activity in rat thymocytes. Intact rat thymocytes (○) or thymocyte membranes (△) were incubated with the indicated concentrations of *S*-nitroso-cysteine in the presence of 10 μM forskolin. Cyclic AMP accumulation and adenylyl cyclase activity were determined as described in Materials and Methods. Data are normalized as percentages of the forskolin-stimulated activity and are presented as means ± S.E. of 3–4 independent experiments. The absolute values for forskolin-stimulated cyclic AMP accumulation and adenylyl cyclase activity were 24.3 ± 3.8 pmol/10^6 cells per 5 min ($n = 4$) and 11.3 ± 1.3 pmol/mg protein per min ($n = 3$), respectively.

3.3. Inhibitory effect of S-nitroso-cysteine on adenylyl cyclase in the membranes and its reversal by dithiothreitol treatment

In membranes from rat thymocytes, *S*-nitroso-cysteine at 20 μM inhibited 10 μM forskolin-stimulated adenylyl cyclase activity significantly, with a maximal inhibition by *S*-nitroso-cysteine occurring at 200 μM (Fig. 3). The ED_{50} value of *S*-nitroso-cysteine for inhibition of membrane adenylyl cyclase activity was 55 ± 15 μM ($n = 4$), which was much higher than the ED_{50} value for intact thymocytes. Adenylyl cyclase activity in the membranes was stimulated by both 10 μM forskolin and 100 μM GTPγS (Table 3). In membranes first incubated with 0.4 mM *S*-nitroso-cysteine for 10 min and then washed with *S*-nitroso-cysteine-free buffer, the basal (non-stimulated), GTPγS- and forskolin-stimulated adenylyl cyclase activity was significantly inhibited. Thus, the effect of *S*-nitroso-cysteine on adenylyl cyclase was not reversed by washing the membranes. Interestingly, the second incubation of the *S*-nitroso-cysteine-treated membranes with dithiothreitol caused significant, but not complete, restoration of GTPγS- and forskolin-stimulated adenylyl cyclase activity. Treating the membranes with 5 mM dithiothreitol had no effect on

Table 3
Reversal of *S*-nitroso-cysteine-induced inhibition of adenylyl cyclase by treatment with dithiothreitol

	Adenylyl cyclase activity (%)			
1st treatment with SNC	−	−	+	+
2nd treatment with DTT	−	+	−	+
None	100	110 ± 5	46 ± 21	118 ± 21
100 μM GTPγS	100	98 ± 1	40 ± 8	77 ± 12[a]
10 μM Forskolin	100	99 ± 1	43 ± 12	77 ± 10[a]

The thymocyte membranes were first incubated with or without 0.4 mM *S*-nitroso-cysteine for 10 min at 30°C, and then the washed membranes were incubated with or without 5 mM dithiothreitol for 10 min at 30°C. After the membranes were washed, the adenylyl cyclase activity of the membranes was determined in the presence of 100 μM GTPγS, 10 μM forskolin or vehicle. SNC, *S*-nitroso-cysteine; DTT, dithiothreitol. Data are normalized as percentages of the activity in the control membranes and are presented as means ± S.E. of 3–4 independent experiments. The absolute values for control, GTPγS- and forskolin-stimulated adenylyl cyclase activity in the control membranes (without treatments) were 2.55 ± 0.29, 13.7 ± 1.5 and 11.3 ± 1.3 pmol/mg protein per min, respectively. Data were analyzed by the unpaired *t*-tests. [a] $P < 0.01$ vs. without secondary dithiothreitol treatment.

basal, GTPγS- or forskolin-stimulated activity. Although the effect of dithiothreitol was not significant because of large variations, secondary treatment with dithiothreitol also restored the basal activity in *S*-nitroso-cysteine-treated membranes. The effect of another reducing agent, 2-mercaptoethanol (5 mM), was limited; treating the membranes with 5 mM 2-mercaptoethanol had no effect on basal, GTPγS- or forskolin-stimulated adenylyl cyclase activity, and the second incubation of the *S*-nitroso-cysteine-treated membranes with 2-mercaptoethanol did not restore adenylyl cyclase activity (basal, 48 ± 11; GTPγS-stimulated, 42 ± 9; forskolin-stimulated, 44 ± 14%, which were almost the same as those without 2-mercaptoethanol).

4. Discussion

4.1. Cyclic GMP accumulation via NO in rat thymocytes

We reported earlier that addition of sodium nitroprusside or $NaNo_2$ to culture medium markedly inhibited concanavalin A-stimulated [^{3}H]thymidine incorporation. Addition of 1 mM dibutyryl cyclic GMP also inhibited concanavalin A-stimulated DNA synthesis significantly (Yamada et al., 1998). These findings suggest that the NO-cyclic GMP system regulates DNA synthesis in rat thymocytes. The addition of NO donors stimulated cyclic GMP accumulation in rat thymocytes. The effects of sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine and *S*-nitroso-cysteine (up to 10 μM) were concentration-dependent (Figs. 1 and 2). The effect of sodium nitroprusside and *S*-nitroso-cysteine seemed to be derived from the release of NO into the assay mixture, because (1) the effects of NO donors except *S*-nitroso-*N*-acetylpenicillamine were completely abolished by oxyhemoglobin (Table 1) and (2) addition of NOC-18, which releases NO selectively in acidic and neutral conditions (Hrabie et al., 1993), also stimulated cyclic GMP (Table 1).

S-Nitroso-*N*-acetylpenicillamine-stimulated cyclic GMP accumulation was not inhibited by oxyhemoglobin. Kowaluk and Fung (1990) previously reported that the release of NO from *S*-nitroso-*N*-acetylpenicillamine is not spontaneous and might be catalyzed by cell membranes. Our finding and this report together suggest that NO is produced by *S*-nitroso-*N*-acetylpenicillamine in intracellular compartments of thymocytes, as suggested for the hippocampus (Satoh et al., 1996a,b). Because the effects of *S*-nitroso-*N*-acetylpenicillamine in other cell types have been shown to be inhibited by oxyhemoglobin, the effects of *S*-nitroso-*N*-acetylpenicillamine may be dependent on the cell type studied.

4.2. Inhibitory effects of S-nitroso-cysteine at higher concentrations on cyclic GMP and cyclic AMP accumulation

S-Nitroso-cysteine at concentrations over 10 μM inhibited cyclic GMP (Fig. 1) and cyclic AMP accumulation (Fig. 3 and Table 2). The inhibitory effects of *S*-nitroso-cysteine on cyclic nucleotides did not seem to be due to NO, because (1) other NO donors including NOC-18 did not inhibit either cyclic AMP or cyclic GMP and (2) high concentrations of *S*-nitroso-cysteine inhibited cyclic GMP in the presence of oxyhemoglobin (1 mg/ml). These findings also exclude the involvement of cyclic GMP in cyclic AMP inhibition and the inhibition of cyclic GMP accumulation by high concentrations of *S*-nitroso-cysteine, at least under our conditions. Addition of *S*-nitroso-cysteine to the assay mixture inhibited the adenylyl cyclase activity of the membranes (Fig. 3). Thus, it is probable that there is not substantial cross-talk between the cyclic GMP and cyclic AMP pathways in these cells, and that *S*-nitroso-cysteine inhibits both cyclic AMP and cyclic GMP accumulation directly.

Mayer et al. (1992) reported that cyclic GMP phosphodiesterase was activated and cyclic GMP formation was apparently inhibited by NO in brain synaptosomes. In our assay mixture, however, three kinds of phosphodiesterase inhibitors at sufficient concentrations were added. It has been suggested that sulfhydryl groups may be critical for the regulation of guanylyl cyclase, and that thiols regulate this activity by redox mechanisms (Niroomand et al., 1989; Murad, 1994; Mayer et al., 1995). Thus, it is likely that *S*-nitroso-cysteine, but not NO, directly interacts and inhibits guanylyl cyclase.

It has been reported that NO donors modulate cytosolic free Ca^{2+} concentrations in some cells (Shin et al., 1992; Mery et al., 1993; Clementi et al., 1996). Some types of adenylyl cyclase are known to be regulated by Ca^{2+}/calmodulin (for review, see Tang and Gilman, 1992). However, Ca^{2+} was not involved in the *S*-nitroso-cysteine-induced inhibition of cyclic AMP accumulation, because removal of extracellular $CaCl_2$ and treatment with a calmodulin antagonist had no effect on *S*-nitroso-cysteine-induced inhibition. Adenylyl cyclase activity in thymocyte membranes in the absence of $CaCl_2$ was inhibited by the addition of *S*-nitroso-cysteine to the assay mixture (Fig. 3). These findings suggest that the inhibition of cyclic AMP accumulation by *S*-nitroso-cysteine is caused by the direct inhibition of adenylyl cyclase in the membranes.

4.3. Inhibition of adenylyl cyclase by S-nitroso-cysteine and its reversal by dithiothreitol treatment

Nitrogen monoxides such as NO interact directly with various protein molecules (for review, see Stamler, 1994) including adenylyl cyclase. Duhe et al. (1994) reported that critical cysteine residues of Ca^{2+}/calmodulin-sensitive type I adenylyl cyclase are modulated by NO and *S*-nitroso-cysteine, thus inhibiting activity, and that dithiothreitol partially restored this activity. However, there are three major differences between our results and their results, namely, the target adenylyl cyclase in thymocytes is

not Ca^{2+}/calmodulin-sensitive, the inhibitory effect on adenylyl cyclase in thymocytes was obtained only with *S*-nitroso-cysteine, and NO is not involved in *S*-nitroso-cysteine-induced inhibition of this activity. The first discrepancy can be explained by the existence of Ca^{2+}/calmodulin-insensitive adenylyl cyclase in rat thymocytes. Because all reported adenylyl cyclases contain cysteine residues (Tang and Gilman, 1992), it is likely that Ca^{2+}/calmodulin-insensitive adenylyl cyclase(s) is the target for *S*-nitroso-cysteine in rat thymocytes. The types of adenylyl cyclase in rat thymocytes are now being investigated. The second and third discrepancies seem to depend on the experimental conditions or cell types used. It is probable that NO reacts with intracellular molecules containing cysteine and resultant molecules such as *S*-nitroso-cysteine exert an inhibiting effect in some cells, but the level of these molecules may be low in thymocytes. In agreement with the results by Duhe et al. (1994), however, the inhibition of adenylyl cyclase activity by *S*-nitroso-cysteine was reversed by treating the thymocyte membranes with dithiothreitol (Table 3).

There are several mechanisms which are regulated by nitrogen monoxides (for review, see Stamler, 1994). NAD-dependent modifications including NAD attachment are probable, as in the case of glyceraldehyde-3-phosphate dehydrogenase (Molina et al., 1992; McDonald and Moss, 1993), and studies have indicated that these modifications are stimulated by *S*-nitrosothiols and related nitrosating agents, rather than by NO itself (Mohr et al., 1994). However, the inhibition of adenylyl cyclase by *S*-nitroso-cysteine was observed in the membranes without NAD in our studies. *S*-Nitrosylation of protein thiols by NO donors is another important regulatory mechanism. Inactivation of glutathione peroxidase (Asahi et al., 1995) and protein kinase C (Gopalakrishna et al., 1993) by NO donors is partially reversed by the addition of dithiothreitol. Because the inhibitory effect of *S*-nitroso-cysteine on adenylyl cyclase was stable and not reversed by washing the membranes, and the addition of dithiothreitol restored the adenylyl cyclase activity in the *S*-nitroso-cysteine-treated membranes, *S*-nitrosylation of the cysteine residues of adenylyl cyclase by *S*-nitroso-cysteine is one of the most plausible mechanisms for its inhibitory activity. Alternatively, *S*-nitroso-cysteine may interfere with disulfide bonds in adenylyl cyclase or GTP-binding proteins. Further studies are needed to determine the mechanisms of action of *S*-nitroso-cysteine on adenylyl (and guanylyl) cyclases.

4.4. Physiological relevance of S-nitrosothiols and S-nitroso-cysteine

It is well known that, in the presence of metals, NO is easily converted into other active molecule. Our results show that *S*-nitroso-cysteine, but not cysteine or NO, is involved in the inhibition of adenylyl cyclase in rat thymocytes. In a preliminary experiment, co-addition of 0.5 mM *S*-nitroso-*N*-acetylpenicillamine and 1 mM cysteine inhibited forskolin-stimulated cyclic AMP accumulation (data not shown). Previously, we reported that co-addition of NO donors (sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine) with thiol agents (dithiothreitol and cysteine) stimulated noradrenaline release from rat hippocampus in vitro and in vivo in a NO/cyclic GMP-independent manner (Satoh et al., 1996a,b). Although NO donors such as sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine by themselves had no effect, *S*-nitroso-cysteine stimulates noradrenaline release without co-factors (Satoh et al., 1997). Myers et al. (1990) reported that the vasorelaxant properties of the endothelium-derived relaxing factor more closely resembled those of *S*-nitroso-cysteine than those of NO. *S*-Nitrosothiol groups in proteins may serve as stable intermediates in the cellular metabolism of NO (Stamler, 1994). *S*-Nitrosothiols including *S*-nitroso-cysteine are formed under physiological conditions by several reaction mechanisms (Kharitonov et al., 1995; Gow et al., 1997). These results and previous reports suggest the formation and biological role of *S*-nitrosothiols under physiological conditions. We reported earlier that the effect of *S*-nitroso-cysteine on noradrenaline release in the hippocampus was inhibited markedly by specific amino acids such as L-leucine and L-alanine, and a specific inhibitor of the L-type amino acid transporter, 2-aminobicyclo[2.2.1]-heptane-2-carboxylate, inhibited the effects of *S*-nitroso-cysteine (Satoh et al., 1997). The effect of *S*-nitroso-cysteine on cyclic GMP accumulation was not inhibited by L-leucine. These findings show that *S*-nitroso-cysteine was incorporated into rat hippocampus via L-type-like amino acid transporters and stimulated noradrenaline release. It is probable that *S*-nitroso-cysteine is incorporated and has direct effects on rat thymocytes, in addition to the generation of NO/cyclic GMP accumulation. The physiological significance of the inhibition of cyclic GMP accumulation and adenylyl cyclase activity by *S*-nitroso-cysteine in rat thymocytes remains to be determined.

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