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Inhibition of phospholipase A_2 activity by *S*-nitroso-cysteine in a cyclic GMP-independent manner in PC12 cells

Siong Heng Thang, Yoshie Yasuda, Michiko Umezawa, Toshihiko Murayama*, Yasuyuki Nomura

Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

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Abstract

Arachidonic acid and nitric oxide (NO) act as retrograde and intercellular messengers in the nervous system. Regulation of cyclooxygenase is well established, but regulation of phospholipase A_2, the enzyme responsible for the liberation of arachidonic acid, by NO has not been thoroughly investigated. Using the PC12 cell line as a neuronal model, we studied the effects of exogenous NO compounds on arachidonic acid release. Incubation with Ca^{2+} ionophores or mastoparan (wasp venom peptide) stimulated [^{3}H]arachidonic acid release from prelabeled PC12 cells. [^{3}H]Arachidonic acid release was inhibited by cytosolic phospholipase A_2 inhibitors, but not by dithiothreitol. A cytosolic phospholipase A_2 protein band with a molecular mass of ~100 kDa was detected by immunoblotting. *S*-Nitroso-cysteine inhibited basal and stimulated [^{3}H]arachidonic acid release in concentration-dependent manners. Other NO compounds such as sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine did not affect [^{3}H]arachidonic acid release. *N*-Ethylmaleimide also inhibited [^{3}H]arachidonic acid release. The inhibitory effects of *S*-nitroso-cysteine and *N*-ethylmaleimide were irreversible, because [^{3}H]arachidonic acid release from PC12 cells preincubated with *S*-nitroso-cysteine or *N*-ethylmaleimide was much lower than that from nontreated cells. These findings suggest (a) cytosolic phospholipase A_2 is activated by Ca^{2+} or mastoparan, and inhibited by *S*-nitroso-cysteine in a cyclic GMP-independent manner, (b) *N*-ethylmaleimide also inhibits cytosolic phospholipase A_2 and arachidonic acid release in PC12 cells. *S*-Nitroso-cysteine can regulate the production of other retrograde messenger arachidonic acid.

Keywords: Arachidonic acid; Cytosolic phospholipase A_2; *S*-Nitroso-cysteine; cGMP; PC12 cell

1. Introduction

Long-term potentiation, which is involved in learning and memory, is triggered postsynaptically and its expression is partially derived from presynaptic mechanisms. The involvement of retrograde messengers that are released from postsynaptic neurons and diffuse back across presynaptic neurons to increase neurotransmitter release has been proposed. Several candidates including arachidonic acid and nitric oxide (NO) have been examined (for a review, see Medina and Izquierdo, 1995). Additionally, brain stress such as ischemia induces a rapid increase in free arachidonic acid (and its metabolites) and free radicals including NO, all of which are potent neuronal injury mediators in the brain (for a review, see Bazan et al., 1995). These findings indicate physiological and pathological roles for arachidonic acid and NO in neuronal tissues.

Phospholipase A_2 catalyzes the hydrolysis of phospholipids at the sn-2 position to produce lysophospholipids and fatty acids such as arachidonic acid (for a review, see Leslie, 1997). Several different forms of phospholipase A_2 exist. Two types of mammalian phospholipase A_2 have recently been purified, cloned and sequenced; the 10–14 kDa secreted phospholipase A_2 and the 85–100 kDa cytosolic phospholipase A_2. Their structural and biochemical properties differ greatly; cytosolic phospholipase A_2 has a marked preference for arachidonic acid esterified at the sn-2 position of phospholipids and is active at submicromolar concentrations of Ca^{2+}, but secreted phospholipase

* Corresponding author. Tel.: +81-11-706-3248; fax: +81-11-706-4987.

E-mail address: murayama@pharm.hokudai.ac.jp (T. Murayama).

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A_2 does not exhibit a fatty acid preference, requires millimolar concentrations of Ca^{2+} for catalytic activity and is inhibited by dithiothreitol treatment. Activation of cytosolic phospholipase A_2 represents the rate-limiting step in the formation of modulators of synaptic transmission such as arachidonic acid and its metabolites (Medina and Izquierdo, 1995). A sustained activation of cytosolic phospholipase A_2 has been reported after ischemia/reperfusion in the brain (Rordorf et al., 1991; Bonventre and Koroshetz, 1993). Bonventre et al. (1997) created ''knockout'' mice that lack cytosolic phospholipase A_2, and reported that the knockout mice had smaller infarcts and developed fewer brain edemas and fewer neurological deficits. These reports illustrate the involvement of cytosolic phospholipase A_2 in various functions in nervous systems. Ca^{2+}-independent phospholipase A_2 activities have also been purified and characterized, but their roles in the brain have not been established (Leslie, 1997). There are several reports showing the regulation and functions of arachidonic acid and NO in PC12 cells. Nerve growth factor (NGF) stimulated arachidonic acid release (Zheng et al., 1996), and an inhibitor of lipooxygenase, which activates arachidonic acid cascade leading to leukotrienes, reduced the induction of neurite outgrowth by NGF in PC12 cells (De George et al., 1988). Cell cycle arrest and differentiation such as neurite outgrowth caused by NGF are associated with induction of NO synthases including inducible type (Peunova and Enikolopov, 1993; Poluha et al., 1997). Inhibition of NO synthase activity has been shown to reverse NGF-induced differentiation. Farinelli et al. (1996) reported that chemical NO compounds such as sodium nitroprusside promoted survival after removal of serum from native PC12 cells and removal of NGF from neuronally differentiated PC12 cells. Further, NO produced by NO compounds through the stimulation of the cyclic GMP pathway stimulated and/or enhanced expression of the immediate early genes c-*fos* and *jun*B (Peunova and Enikolopov, 1993; Haby et al., 1994) as well as the activity of tyrosine hydroxylase (Roskoski and Roskoski, 1987). These reports show that arachidonic acid and NO regulate various cell functions in PC12 cells. We previously reported that ATP receptor stimulation enhances Ca^{2+}-induced arachidonic acid release (Murayama et al., 1995). However, the type of phospholipase A_2 involved in arachidonic acid release from PC12 cells has not been established, and the interactions between arachidonic acid release and NO in neuronal cells including PC12 cells have not been studied. The aim of the present study was to investigate the regulation of arachidonic acid release by NO compounds in PC12 cells. The findings show that (1) arachidonic acid release is mediated by cytosolic phospholipase A_2, and (2) arachidonic acid release is irreversibly inhibited by both *S*-nitroso-cysteine and by *N*-ethylmaleimide. The involvement of *S*-nitrosylation by *S*-nitroso-cysteine or alkylation by *N*-ethylmaleimide of Cys residues in cytosolic phospholipase A_2 is discussed.

2. Materials and methods

2.1. Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (215 Ci/mmol, 7.96 TBq/mmol) was purchased from Amersham (Buckinghamshire, England). Mastoparan, *N*-ethylmaleimide, ionomycin, A23187 and 4-bromophenacyl bromide were purchased from Sigma (St. Louis, MO, USA). *S*-Nitroso-cysteine and 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC-18) were purchased from Dojindo Lab. (Kumamoto, Japan). *S*-Nitroso-*N*-acetylpenicillamine and arachidonyl trifluoromethyl ketone were purchased from Research Biochemicals (Natick, MA, USA). Sodium nitroprusside, $NaNO_2$ and mepacrine were purchased from Wako (Osaka, Japan). 3-Morpholinosydonimine and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, an inhibitor of guanylyl cyclase were obtained from BIOMOL (PA, USA) and Tocris (Britol, UK), respectively.

2.2. Cell culture and measurement of [^{3}H]arachidonic acid release

PC12 cells were cultured on collagen-coated dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 5% horse serum, as previously reported (Murayama et al., 1995, 1996; Naganuma et al., 1998). [^{3}H]Arachidonic acid release from prelabeled PC12 cells was determined as described previously (Murayama et al., 1995). In brief, PC12 cells on dishes were incubated with Dulbecco's modified Eagle's medium (0.2% serum) and 1 μCi/ml (37 kBq/ml) of [^{3}H]arachidonic acid for 24 h. The labeled cells were detached from dishes and washed twice by centrifugation ($200 \times g$, 2 min) at 4°C and resuspended in a modified Tyrode HEPES buffer (137 mM NaCl, 1 mM Na_2HPO_4, 12 mM $NaHCO_3$, 3 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES (pH 7.4)). Cell suspensions (40–60 μg protein) were stimulated with the indicated additions for 20 min at 37°C. The assay buffer was further supplemented with 0.2% fatty acid free bovine serum albumin and the total assay volume was 200 μl. The reaction was terminated by adding 500 μl of ice-cold, Ca^{2+}-, Mg^{2+}- and albumin-free Tyrode HEPES buffer containing 5 mM EDTA and 5 mM EGTA and then centrifuged ($8000 \times g$, 30 s). The ^{3}H content of the supernatant (release of nonesterified [^{3}H]arachidonic acid and ^{3}H-containing metabolites) was estimated by liquid scintillation spectrometry. Data are calculated as percentages relative to the total incorporation of [^{3}H]arachidonic acid. We estimated the radioactivity in the supernatant as [^{3}H]arachidonic acid release without separation, because the rate-limiting step in the synthesis of metabolites appears to be the release of arachidonic acid from phospholipids (Medina and Izquierdo, 1995). The metabolites of

arachidonic acid in the supernatant were analyzed separately using thin-layer chromatography as described previously (Murayama and Ui, 1987). The radioactivities of arachidonic acid and its metabolites were reduced by *S*-nitroso-cysteine at a similar rate.

2.3. Leakage of lactate dehydrogenase

PC12 cell viability was measured by leakage of lactate dehydrogenase as described (Yoshinaga et al., 1998).

2.4. Immunoblot analysis

Immunoblotting was conducted as described previously (Kramer et al., 1996) with some modifications. The total homogenates of PC12 cells were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred at 4°C to nitrocellulose membranes (Bio-Rad). The membranes were incubated with the first antibody (anti-human cytosolic phospholipase A_2 antibody, N-216, Santa Cruz Biotechnology, CL, USA; diluted to 1:2000) followed by secondary antibody (horseradish peroxidase-linked antibody, Amersham, diluted to 1:2000). Peroxidase was detected by chemiluminescence using the enhanced chemiluminescence detection system (ECL kit, Amersham).

2.5. Statistics

Data are the mean ± S.E. of three to six independent experiments done with different cell cultures, and were analyzed using the unpaired *t* test. In the case of multiple comparisons, significance of differences was determined using one-way analysis of variance followed by Dunnett's or Tukey test. *P*-values of < 0.01 were considered to be significant. In some experiments, data are the mean ± S.D. of three determinations in a typical experiment and are representative of two independent experiments.

3. Results

3.1. Mastoparan-stimulated [^{3}H]arachidonic acid release in PC12 cells

Previously, we reported that mastoparan, a wasp venom toxin, increased $[Ca^{2+}]_i$ levels from intracellular Ca^{2+} pools and induced noradrenaline release in PC12 cells (Murayama et al., 1996). In the present study, we examined the effect of mastoparan on [^{3}H]arachidonic acid release. Although Choi et al. (1992) reported that mastoparan had no effect on arachidonic acid release in PC12 cells, the addition of 20 μM mastoparan remarkably stimulated [^{3}H]arachidonic acid release from the prelabeled PC12 cells (D-type) used in the present study (Fig. 1 and Table 1). Addition of 10 μM mastoparan also stimulated [^{3}H]arachidonic acid release. In a previous report concerning noradrenaline release (Murayama et al., 1996), the effect of mastoparan on [^{3}H]arachidonic acid release was not dependent on extracellular $CaCl_2$. The net increases caused by 20 μM mastoparan were 8.9 ± 0.3 and 9.2 ± 0.4 (% of total, $n = 3$) in the absence or presence of 1 mM

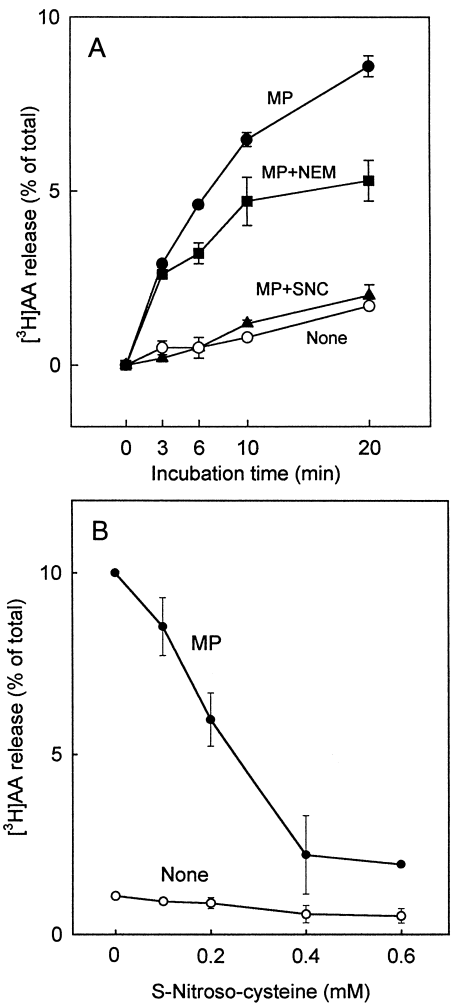

Fig. 1. Mastoparan-stimulated [^{3}H]arachidonic acid release and its inhibition by *S*-nitroso-cysteine and *N*-ethylmaleimide in PC12 cells. (A) PC12 cells prelabeled with [^{3}H]arachidonic acid were stimulated for the indicated time with 20 μM mastoparan (●, ▲, ■) or vehicle (○). The assay mixture was further supplemented with 0.4-mM *S*-nitroso-cysteine (SNC, ▲) or 0.4-mM *N*-ethylmaleimide (NEM, ■) in the presence of mastoparan. Data are presented as percentages of the total incorporated [^{3}H]arachidonic acid, and are the mean ± S.D. of three determinations in a typical experiment. Similar data were obtained from two independent experiments. (B) PC12 cells prelabeled with [^{3}H]arachidonic acid were incubated for 20 min with the indicated concentrations of *S*-nitroso-cysteine in the presence (●) or absence (○) of 20-μM mastoparan. Data are presented as percentages of the total incorporated [^{3}H]arachidonic acid, and are the mean ± S.E. of three to six independent experiments.

Table 1
Effects of phospholipase A_2 inhibitors on mastoparan-stimulated [^{3}H]arachidonic acid release in PC12 cells
PC12 cells prelabeled with [^{3}H]arachidonic acid were preincubated with the indicated concentrations of mepacrine (EXPERIMENT I), 4-bromophenacyl bromide (BPB), arachidonyl trifluoromethyl ketone ($AACOCF_3$), diacylglycerol lipase inhibitor (RHC 80267) (EXPERIMENT II) or vehicle for 10 min. Then the washed cells were stimulated with 10 or 20 μM mastoparan for 20 min in the presence of the same concentrations of PLA2 inhibitors or RHC 80267. Data are presented as percentages of the total incorporated [^{3}H]arachidonic acid, and are the mean ± S.D. of three determinations in a typical experiment. Similar data were obtained from two independent experiments.

Addition	[^{3}H]Arachidonic acid release (% of total)	
	None	Mastoparan
Experiment I		
10 μM Mastoparan	1.45 ± 0.32	6.49 ± 0.41
+ Mepacrine (30 μM)	1.83 ± 0.34	3.93 ± 0.22
Experiment II		
20 μM Mastoparan	1.95 ± 0.24	9.61 ± 0.47
+ BPB (50 μM)	2.06 ± 0.27	5.06 ± 0.43
+ BPB (100 μM)	2.85 ± 0.38	4.07 ± 0.54
+ $AACOCF_3$ (10 μM)	2.45 ± 0.34	3.46 ± 0.31
+ RHC 80267 (10 μM)	1.91 ± 0.23	9.21 ± 0.61

$CaCl_2$, respectively. In subsequent experiments, [^{3}H]arachidonic acid release was assayed in the presence of 1 mM $CaCl_2$. [^{3}H]Arachidonic acid release by 20 μM mastoparan was not the result of cell toxicity. The reasons were (1) lactate dehydrogenase activity in supernatant fraction of PC12 cells treated with 20 μM mastoparan was under 3%, which was similar to that in control cells, and (2) [^{3}H]arachidonic acid release caused by mastoparan was inhibited by PLA_2 inhibitors (Table 1) as mentioned below.

3.2. [^{3}H]Arachidonic acid release by cytosolic phospholipase A_2 activation in PC12 cells

The stimulatory effect of 10 μM mastoparan on [^{3}H]arachidonic acid release was inhibited by 30 μM mepacrine, an inhibitor of phospholipase A_2 (Kajiyama et al., 1989; Tapia-Arancibia et al., 1992), as shown in Table 1. 4-Bromophenacyl bromide, another inhibitor of phospholipase A_2 (Yokokawa et al., 1989; Tapia-Arancibia et al., 1992) also inhibited the effect of 20 μM mastoparan. Arachidonyl trifluoromethyl ketone is a specific inhibitor of cytosolic phospholipase A_2 and its inhibition of other types of phospholipase A_2 is more than 1000-fold less (Bartoli et al., 1994). Treatment for 10 min before an assay with 10 μM arachidonyl trifluoromethyl ketone, the concentration at which it inhibits cytosolic phospholipase A_2 but not secreted phospholipase A_2 nor Ca^{2+}-independent phospholipase A_2 (Ackermann et al., 1995; Atsumi et al., 1998), almost completely inhibited 20 μM mastoparan-stimulated [^{3}H]arachidonic acid release. The net increase by 20 μM mastoparan in 10 μM arachidonyl trifluoromethyl ketone-treated cells was 13% of the net increase in control cells. Treatment with 10 μM diacylglycerol lipase inhibitor (RHC 80267) did not affect the level of mastoparan-stimulated [^{3}H]arachidonic acid release. Although secreted phospholipase A_2 is inhibited by reducing agents which split the intramolecular disulfide bonds essential for activity, cytosolic phospholipase A_2 is not inhibited by dithiothreitol (Clark et al., 1990). [^{3}H]Arachidonic acid release from PC12 cells was not modified by 5 mM dithiothreitol; the basal (non-stimulated) release was 1.85 ± 0.35 and 2.18 ± 0.13 (% of total, $n = 3$), in the absence and presence of 5 mM dithiothreitol, and the release by 20 μM mastoparan was 8.82 ± 0.24 and 8.47 ± 0.59% ($n = 3$), in the absence and presence of dithiothreitol. These findings suggest that [^{3}H]arachidonic acid release by mastoparan from PC12 cells is via activation of cytosolic phospholipase A_2. In this respect, we detected an immunoreactive band (about 100 kDa), which cross-reacted with anti-cytosolic phospholipase A_2 antibody, in total homogenate of PC12 cells (Fig. 2).

3.3. Inhibition of [^{3}H]arachidonic acid release by S-nitroso-cysteine, but not by other NO compounds, in PC12 cells

Next we investigated the effects of various NO compounds on [^{3}H]arachidonic acid release from PC12 cells (Table 2). Addition of 0.5 mM *S*-nitroso-cysteine significantly inhibited basal and 20 μM mastoparan-stimulated [^{3}H]arachidonic acid release. As shown in Fig. 1A, 0.4 mM *S*-nitroso-cysteine inhibited [^{3}H]arachidonic acid release immediately after its addition. The inhibition by *S*-nitroso-cysteine was concentration-dependent and significant at concentrations as low as 0.2 mM (Fig. 1B). The addition of *S*-nitroso-cysteine at concentrations higher than 0.6 mM slightly stimulated [^{3}H]arachidonic acid release. We investigated the inhibitory effects of lower concentrations of *S*-nitroso-cysteine, because it was difficult to distinguish whether the effect of high concentrations of *S*-nitroso-cysteine was due to toxicity. The prospective EC_{50} value of *S*-nitroso-cysteine by computerized analysis

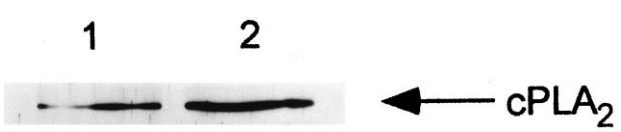

Fig. 2. Immunoblot analysis of cytosolic phospholipase A_2 in PC12 cells. The total homogenates of PC12 cells (2 μg, lane 1 and 4 μg of protein, lane 2) were subjected to SDS-PAGE followed by immunoblot analysis using anti-cytosolic phospholipase A_2 antibody. Data are typical of three independent experiments.

Table 2
Effects of various NO compounds on [^{3}H]arachidonic acid release in PC12 cells
PC12 cells prelabeled with [^{3}H]arachidonic acid were incubated with or without 20 μM mastoparan in the presence of various NO compounds (0.5 mM) for 20 min. 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. Data are presented as percentages of the total incorporated [^{3}H]arachidonic acid, and are the mean ± S.E. of three to five independent experiments.

Addition	[^{3}H]AA release (% of total)	
	None	20 μM Mastoparan
None	1.27 ± 0.16	9.72 ± 0.69
0.5 mM SNC	0.31 ± 0.05[a]	1.83 ± 0.75[a]
0.5 mM SNP	0.84 ± 0.05	8.28 ± 1.37
0.5 mM SNAP	0.86 ± 0.06	7.82 ± 1.36
0.5 mM $NaNO_2$	1.06 ± 0.06	9.23 ± 1.59
0.5 mM NOC-18	Not determined	8.12 ± 0.91

[a] $P < 0.01$, significantly different compared with no treatment.

was 0.24 ± 0.08 mM ($n = 4$). Addition of 0.6 mM L-cysteine had no effect on [^{3}H]arachidonic acid release. Addition of 0.5 mM *S*-nitroso-*N*-acetylpenicillamine slightly, but not significantly, inhibited basal and mastoparan-stimulated [^{3}H]arachidonic acid release. NOC-18, a specific releaser of NO radical, also had a slight inhibitory effect. However, even at higher concentrations (0.8 mM), *S*-nitroso-*N*-acetylpenicillamine and NOC-18 did not significantly inhibit basal and mastoparan-stimulated [^{3}H]arachidonic acid release. Neither 0.5 mM sodium nitroprusside nor $NaNO_2$ modified basal or mastoparan-stimulated [^{3}H]arachidonic acid release. Previously, we reported that *S*-nitroso-*N*-acetylpenicillamine, sodium nitroprusside and NOC-18 stimulated cyclic GMP accumulation in a concentration-dependent manner, reaching a maximum 300 μM in PC12 cells (Naganuma et al., 1998). The addition of *S*-nitroso-cysteine from 3 to 30 μM markedly stimulated cyclic GMP accumulation, but *S*-nitroso-cysteine over 30 μM to 0.4 mM inhibited the accumulation of cyclic GMP, while the cyclic GMP level after stimulation with 0.3 mM *S*-nitroso-cysteine was 10 times higher than the basal level (Naganuma et al., 1998). Co-addition of 0.5 mM 8-bromo cyclic GMP or dibutyryl cyclic GMP did not inhibit basal and mastoparan-stimulated [^{3}H]arachidonic acid release (data not shown). Also, the addition of 0.1 mM 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, a selective inhibitor of NO radical-sensitive guanylyl cyclase, did not modify the inhibitory effect of *S*-nitroso-cysteine. These findings suggest that cyclic GMP does not mediate the inhibitory effect of *S*-nitroso-cysteine on arachidonic acid release. In the presence of oxyhemoglobin (500 μg/ml), an NO radical scavenger (Satoh et al., 1996), 20 μM mastoparan-stimulated [^{3}H]arachidonic acid release was inhibited by 0.5 mM *S*-nitroso-cysteine to 2.01 ± 0.77%, which is a similar level when in the absence of oxyhemoglobin. In the presence of 0.5 mM 3-morpholinosydnomine, which decomposes to generate both NO radicals and O_2^- and provide a continuous source of peroxynitrite, [^{3}H]arachidonic acid release by 20 μM mastoparan was 8.8 ± 0.4 (% of total) in a typical experiment. In the absence of extracellular $CaCl_2$, similar findings showing concentration-dependent inhibition of [^{3}H]arachidonic acid release by *S*-nitroso-cysteine, but not by other NO compounds, were obtained (data not shown). As previously reported (Murayama et al., 1995), addition of Ca^{2+} ionophore (10 μM ionomycin and 20 μM A23187) stimulated [^{3}H]arachidonic acid release in the presence of 1 mM extracellular $CaCl_2$ (Table 3). [^{3}H]Arachidonic acid release by Ca^{2+} ionophore was markedly inhibited by phospholipase A_2 inhibitor (30 μM mepacrine, 100 μM 4-bromophenacyl bromide) and by a cytosolic phospholipase A_2 inhibitor (10 μM arachidonyl trifluoromethyl ketone), but not by 5 mM dithiothreitol. In a typical experiment, 10 μM ionomycin-stimulated [^{3}H]arachidonic acid release was 4.55 ± 0.50 and 1.40 ± 0.38 (% of total) from control and arachidonyl trifluoromethyl ketone-treated PC12 cells. Addition of 0.4 mM *S*-nitroso-cysteine inhibited both the effects of ionomycin and A23187. The inhibitory effect of *S*-nitroso-cysteine is irreversible, because [^{3}H]arachidonic acid release from PC12 cells preincubated with 0.4 mM *S*-nitroso-cysteine for 5 min and washed by centrifugation was significantly lower than that from non-treated PC12 cells (Table 4).

3.4. Inhibition of mastoparan-stimulated [^{3}H]arachidonic acid release by N-ethylmaleimide in PC12 cells

Cytosolic phospholipase A_2, which contains nine Cys residues, is stable in the presence of reducing agents (Leslie, 1997). Although the Cys residues are not essential for the activity, sulfhydryl-modifying reagents such as iodoacetamide and *N*-ethylmaleimide react with Cys^{331} and inactivate cytosolic phospholipase A_2, which suggests that Cys^{331} is located near the active site (Li et al., 1996;

Table 3
Inhibition of Ca^{2+} ionophore-stimulated [^{3}H]arachidonic acid release by *S*-nitroso-cysteine or *N*-ethylmaleimide in PC12 cells
PC12 cells prelabeled with [^{3}H]arachidonic acid were stimulated with the indicated concentrations of Ca^{2+} ionophores in the presence of vehicle (None), 0.4 mM *S*-nitroso-cysteine (SNC) or 0.3 mM *N*-ethylmaleimide (NEM). Data are the mean ± S.D. of three determinations in a typical experiment and are representative of two independent experiments.

Addition	[^{3}H]Arachidonic acid release (% of total)		
	None	0.4 mM SNC	0.3 mM NEM
None	1.25 ± 0.33	0.58 ± 0.04	1.80 ± 0.23
10 μM Ionomycin	4.26 ± 0.45	0.38 ± 0.25	2.01 ± 0.30
20 μM A23187	3.45 ± 0.20	0.89 ± 0.18	Not determined

Pickard et al., 1996). Previously, we reported that *N*-ethylmaleimide caused sustained increase of cytosolic free Ca^{2+} concentration and noradrenaline release in intact PC12 cells (Naganuma et al., 1999). Addition of 0.3 mM *N*-ethylmaleimide in an assay mixture inhibited [^{3}H]arachidonic acid release by mastoparan after ~3 min of lag time (Fig. 1A). Although the basal (non-stimulated) [^{3}H]arachidonic acid release for a 20-min period was not modified by *N*-ethylmaleimide (1.85 ± 0.35 and 1.78 ± 0.25 (% of total, $n = 3$) in the absence and presence of 0.3 mM *N*-ethylmaleimide), [^{3}H]arachidonic acid release by 20 μM mastoparan was significantly inhibited by 0.3 mM *N*-ethylmaleimide; 8.82 ± 0.24 and 5.12 ± 0.60% ($n = 3$), in the absence and presence of *N*-ethylmaleimide. Addition of 0.3 mM *N*-ethylmaleimide inhibited ionomycin-stimulated [^{3}H]arachidonic acid release (Table 3). The effect of *N*-ethylmaleimide did not appear to be derived from cell toxicity, because simultaneous addition of 5 mM dithiothreitol into an assay mixture reacted with *N*-ethylmaleimide and abolished the inhibitory effect of *N*-ethylmaleimide. The [^{3}H]arachidonic acid release from PC12 cells preincubated with 0.2 mM *N*-ethylmaleimide for 5 min and washed by centrifugation was much lower than that from non-treated PC12 cells (Table 4). Thus, the inhibitory effect of *N*-ethylmaleimide was irreversible. Additionally, the degree of inhibition by pretreatment with *N*-ethylmaleimide was much higher than the inhibition by addition of *N*-ethylmaleimide in the assay mixture (Table 4). It is probable that the incorporation of *N*-ethylmaleimide into cells and/or the interaction of *N*-ethylmaleimide with cytosolic phospholipase A_2 requires a significant amount of time.

Table 4
Irreversible inhibition of [^{3}H]arachidonic acid release by *S*-nitroso-cysteine and by *N*-ethylmaleimide in PC12 cells

PC12 cells prelabeled with [^{3}H]arachidonic acid were incubated with 0.4 mM *S*-nitroso-cysteine (SNC), 0.2 mM *N*-ethylmaleimide (NEM) or vehicle (None) for 5 min. The washed cells were stimulated with or without 20 μM mastoparan for 20 min in the absence of SNC and NEM. Data are normalized as percentages of a net increase of [^{3}H]arachidonic acid release by mastoparan from the non-treated (control) cells. Data involving SNC treatment are presented as the mean ± S.E. of three independent experiments. Data for NEM treatment are the results of two independent experiments. The absolute increase in [^{3}H]arachidonic acid after 20 μM mastoparan addition from the control cells was 7.3 ± 0.3% of the total incorporated [^{3}H]arachidonic acid ($n = 5$). The total incorporated amounts of [^{3}H]arachidonic acid in the cells treated with SNC and NEM were similar to that in the control cells. The basal (non-stimulated) [^{3}H]arachidonic acid release was 0.8 ± 0.2% ($n = 3$), 0.6 ± 0.2% ($n = 3$) and 0.4–0.5% ($n = 2$) of the total incorporated [^{3}H]arachidonic acid in the control, SNC-treated and NEM-treated cells, respectively.

Treatment	[^{3}H]Arachidonic acid release by 20 μM mastoparan (%)
None	100
0.4 mM SNC	32.6 ± 16.3[a]
0.2 mM NEM	12.5, 1.3

[a] $P < 0.01$, significantly different compared with no treatment.

4. Discussion

4.1. Involvement of cytosolic phospholipase A_2 in [^{3}H]arachidonic acid release from PC12 cells

Activation of phospholipase A_2, which releases arachidonic acid from phospholipids, represents the rate-limiting step in the biosynthesis of different eicosanoids. Among the multiple forms of phospholipase A_2 expressed in mammalian tissues, cytosolic phospholipase A_2 is thought to mediate hormone or transmitter-regulated arachidonic acid release (Leslie, 1997). Several studies have shown that stimulants such as NGF and a Ca^{2+} ionophore increase arachidonic acid release from PC12 cells (Murayama et al., 1995; Zheng et al., 1996). However, the type of phospholipase A_2 in PC12 cells has not been studied. Findings of the present study show that activation of cytosolic phospholipase A_2, but not secreted phospholipase A_2, is involved in [^{3}H]arachidonic acid release by mastoparan in PC12 cells, because (1) [^{3}H]arachidonic acid release was inhibited by phospholipase A_2 inhibitors, and by a specific cytosolic phospholipase A_2 inhibitor, arachidonyl trifluoromethyl ketone, and (2) dithiothreitol, an inhibitor of secreted phospholipase A_2, did not inhibit [^{3}H]arachidonic acid release. Also Ca^{2+} ionophore-stimulated [^{3}H]arachidonic acid release was inhibited by phospholipase A_2 inhibitors but not by dithiothreitol, as described in Section 3. These findings suggest the involvement of cytosolic phospholipase A_2 in arachidonic acid release from PC12 cells, although we could not exclude the involvement of Ca^{2+}-independent phospholipase A_2. In addition, a protein band, which reacts with anti-cytosolic phospholipase A_2 antibody, was detected in PC12 cells (Fig. 2). Activity of cytosolic phospholipase A_2 is regulated by both, Ca^{2+} levels and phosphorylation of amino acid residues (Leslie, 1997). In our experiments, Ca^{2+} ionophores stimulated [^{3}H]arachidonic acid release in PC12 cells. Although mastoparan stimulated [^{3}H]arachidonic acid release in the absence of extracellular $CaCl_2$, we previously reported that mastoparan stimulated Ca^{2+} mobilization from intracellular Ca^{2+} pools in PC12 cells (Murayama et al., 1996). Thus, mastoparan and Ca^{2+} ionophores appear to increase intracellular Ca^{2+} concentrations and activate cytosolic phospholipase A_2 in PC12 cells. Phosphorylation by mitogen-activated protein kinases is important for the activation of cytosolic phospholipase A_2 in many cells, and protein kinase C and A also can phosphorylate cytosolic phospholipase A_2 in vitro, and cytosolic phospholipase A_2 is also phosphorylated on tyrosine residues by cytokines (Leslie, 1997). In our experiments, however, a decrease in the electrophoretic mobility of cytosolic phospholipase A_2 indicating phosphorylation was not observed in mastoparan-stimulated PC12 cells (data not shown). We previously reported that phorbol 12-myristate 13-acetate, an activator of protein kinase C,

had no effect on [^{3}H]arachidonic acid release (Murayama et al., 1995) and that mastoparan inhibited cyclic AMP accumulation in PC12 cells (Murayama et al., 1996). Thus, the involvement of protein kinase C and kinase A in arachidonic acid release is unlikely. Although phosphorylation by p38 kinase is not required for thrombin receptor-mediated arachidonic acid release in platelets (Kramer et al., 1996), the involvement of phosphorylation and/or other mechanisms in arachidonic acid release by mastoparan remains to be determined.

4.2. Inhibition of cytosolic phospholipase A_2 by S-nitroso-cysteine in PC12 cells

Recent evidence has implicated NO as a major synaptic signaling agent in the nervous system. NO is a highly diffusible gas with a short half-life and is highly reactive with oxygen, superoxide and heme. NO and its derivatives also react with thiol groups of proteins and glutathione to form *S*-nitrosothiols (for a review, see Stamler, 1994). The form of NO which is active in the tissues/cells is unclear. As mentioned in the Introduction, NO compounds caused several physiological effects via NO radical and cyclic GMP in PC12 cells (Peunova and Enikolopov, 1993; Haby et al., 1994; Farinelli et al., 1996). Our present findings, however, show that the inhibitory effect on [^{3}H]arachidonic acid release by *S*-nitroso-cysteine was not via NO radicals or cyclic GMP. The reasons for this are (1) NO compounds such as sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine, which stimulated cyclic GMP levels via NO radicals in PC12 cells (Naganuma et al., 1998), did not inhibit [^{3}H]arachidonic acid release, (2) NOC-18 did not have a significant effect, (3) co-addition of oxyhemoglobin (500 μg/ml), which almost completely inhibits cyclic GMP production by NO radicals in brain slices (Satoh et al., 1996), had no effect on the inhibition of [^{3}H]arachidonic acid release by *S*-nitroso-cysteine, (4) co-addition of 0.5 mM 8-bromo cyclic GMP or dibutyryl cyclic GMP did not inhibit [^{3}H]arachidonic acid release, (5) an inhibitor of guanylyl cyclase did not modify the effect of *S*-nitroso-cysteine, as mentioned in Section 3. Since [^{3}H]arachidonic acid release from PC12 cells was not modified by 3-morpholinosydnomine, which is known to yield peroxynitrite, peroxynitrite does not appear to regulate cytosolic phospholipase A_2.

In this study, *S*-nitroso-cysteine, but not other NO compounds such as sodium nitroprusside, inhibited [^{3}H]arachidonic acid release (Table 2). Since NO is diffusible and reactive with other molecules, why other NO compounds did not affect on arachidonic acid release is unclear. Previously, we reported that coaddition of NO compounds (sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine) with thiol agents (dithiothreitol and cysteine) (Satoh et al., 1996) and *S*-nitroso-cysteine (Satoh et al., 1997) stimulated noradrenaline release from the rat hippocampus in vitro and in vivo. In addition, we also reported that *S*-nitroso-cysteine regulated noradrenaline release (Naganuma et al., 1998) and Ca^{2+} mobilization from intracellular Ca^{2+} pools (Naganuma et al., 1999) in PC12 cells and that *S*-nitroso-cysteine inhibited the activity of adenylyl cyclase in rat thymocytes (Miyakoshi et al., 1998). In these studies, *S*-nitrosothiols including *S*-nitroso-cysteine, but not other NO compounds, showed the effects. *S*-Nitrosothiols are formed in physiological conditions (Gow et al., 1997) and may serve as intermediates in tissues/cells for NO (Stamler, 1994). The concentrations and its regulation of *S*-nitrosothiols in living cells should be determined in future studies. It is probable that NO and *S*-nitrosothiols have a different spectra for the substrates.

4.3. Possible mechanisms of cytosolic phospholipase A_2 inhibition by S-nitroso-cysteine and N-ethylmaleimide

Activity of cytosolic phospholipase A_2 in human monocytic cell line U937 was inactivated by *N*-ethylmaleimide and other thiol reagents (Li et al., 1994). Modification of Cys^{331} is responsible for the inactivation by thiol reagents (Li et al., 1996; Pickard et al., 1996). In our experiments, addition of 0.3 mM *N*-ethylmaleimide to an assay mixture inhibited [^{3}H]arachidonic acid release by mastoparan (Fig. 1A) and by ionomycin (Table 3). The effect of *N*-ethylmaleimide was irreversible in PC12 cells (Table 4), as previously reported for cytosolic phospholipase A_2 enzyme activity (Li et al., 1994). These findings show that in PC12 cells Cys residues of cytosolic phospholipase A_2 may regulate enzyme activity. It is well established that NO reacts with Cys residues and induces *S*-nitrosylation of various enzymes and proteins (Stamler, 1994). In our cytosolic phospholipase A_2 assay system using intact PC12 cells, *S*-nitroso-cysteine inhibited [^{3}H]arachidonic acid release by stimulants in an irreversible manner (Table 4). These findings and reports suggest the *S*-nitrosylation of cytosolic phospholipase A_2 by *S*-nitroso-cysteine and the alkylation by *N*-ethylmaleimide in PC12 cells. The activity of 5,5′-dithiobis(2-nitrobenzoic acid)-inactivated cytosolic phospholipase A_2 was completely recovered on incubation with dithiothreitol (Li et al., 1994), and the inhibition of adenylyl cyclase activity by *S*-nitroso-cysteine in thymocyte membranes was at least partially recovered on incubation with dithiothreitol (Miyakoshi et al., 1998). However, the inhibitory effect of *N*-ethylmaleimide (0.2 mM) or *S*-nitroso-cysteine (0.4 mM) on mastoparan-stimulated [^{3}H]arachidonic acid release was not affected in PC12 cells pretreated with 5 mM dithiothreitol for 20 min (data not shown). The effects of *S*-nitroso-cysteine on the purified cytosolic phospholipase A_2 activity, including reversibility of the activity, needs further analysis.

4.4. Regulation of arachidonic acid release and metabolism by NO

Due to the increases in free fatty acids, eicosanoids and products of lipid peroxidation, phospholipase A_2 has been

proposed as one of the factors responsible for neuronal cell death after brain ischemia (for a review, see Bazan, 1989), and to be essential for apoptosis in several cell types (Agarwal et al., 1993; Jäättelä et al., 1995). Additionally, cytosolic phospholipase A_2 is crucial for the cytotoxic action of tumor necrosis factor in the L925 cell line (Hayakawa et al., 1993). Clemens et al. (1996) reported that a marked induction of cytosolic phospholipase A_2 was observed in activated microglia and astrocytes in the hippocampus 72 h after ischemia. There is also increasing evidence that inducible NO synthase is induced after ischemia in primary astrocytes (Endoh et al., 1994; Iadecola et al., 1995), and that NO is involved in neuronal cell death (Boje and Arora, 1992; Hewett et al., 1994). These studies demonstrate that both arachidonic acid and NO play important pathological roles in cell death. There are several reports showing regulation of arachidonic acid metabolism by NO. It is reported that *N*-methyl-D-aspartate receptor-, but not quisqualate receptor-, mediated arachidonic acid release is inhibited by endogenous NO compounds in cultured neurons (Rodriguez-Alvarez et al., 1996). In this case, NO modulates arachidonic acid release by direct blockade of the *N*-methyl-D-aspartate receptor as proposed previously (Lei et al., 1992), because quisqualate receptor-mediated arachidonic acid release was not modified by NO. We believe that our report is the first showing the regulation of arachidonic acid release mediated by cytosolic phospholipase A_2 activity by a NO compound, *S*-nitroso-cysteine. Cyclooxygenase converts arachidonic acid to the prostaglandins, prostacyclin and thromboxane A_2. It is established that NO increases cyclooxygenase I and II activities (for a review, see Salvemini, 1997). Although the active species of NO and the molecular mechanism of modification by NO may be different, NO seems to regulate arachidonic acid metabolism at two steps, inhibition of phospholipase A_2 activity (arachidonic acid release from phospholipids) and stimulation of cyclooxygenase activity (prostaglandins biosynthesis from arachidonic acid). Characterization of how cytosolic phospholipase A_2 is modified by *S*-nitroso-cysteine and the physiological/pathological contribution of NO-regulated arachidonic acid metabolism to cell function needs further study.

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