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Involvement of the system L amino acid transporter on uptake of *S*-nitroso-L-cysteine, an endogenous *S*-nitrosothiol, in PC12 cells

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Abstract

Previously, we proposed that *S*-nitroso-L-cysteine, an endogenous *S*-nitrosothiol, was incorporated via the system L-like amino acid transporter(s) in rat brain slices. In this study, we investigated the effect of *S*-nitroso-L-cysteine on L-[^{3}H]leucine uptake in PC12 cells (a neuronal cell line). L-[^{3}H]Leucine uptake in PC12 cells was Na^+ independent and significantly inhibited by an inhibitor of system L and by L-phenylalanine, L-cysteine, L-methionine and L-leucine at 1 mM. The effects of L-alanine, L-serine and L-threonine were limited. *S*-Nitroso-L-cysteine, but not other nitric oxide compounds, inhibited L-[^{3}H]leucine uptake, and this inhibitory effect was eliminated by washing with buffer. System L is composed of the 4F2 light chains (LAT1 or LAT2) and the heavy chain, and the transcripts of these components were detected in RNA from PC12 cells. These findings suggest that *S*-nitroso-L-cysteine is incorporated via the system L amino acid transporter and thus regulates cell responses in PC12 cells.

Keywords: Nitric oxide (NO); *S*-Nitroso-L-cysteine; Amino acid transporter; System L; PC12 cell

1. Introduction

Nitric oxide is a biological messenger that plays important roles in physiological and pathophysiological conditions in various tissues (Murad, 1998). The transfer of nitric oxide to the sulfhydryl group(s) of proteins, known as *S*-nitrosylation, is increasingly becoming recognized as one of mechanisms regulating the functions and enzyme activities of proteins in various biological systems (Stamler et al., 1997; Broillet, 1999; Wang et al., 2000). *S*-Nitrosothiols have been identified in a variety of tissues, and many physiological and biological effects induced by *S*-nitrosothiols have been identified (Gow et al., 1997; Broillet, 1999; Wang et al., 2000).

Previously, we reported that *S*-nitroso-L-cysteine, an endogenous *S*-nitrosothiol, stimulated noradrenaline release in rat hippocampus in vivo (Satoh et al., 1997) and in rat hippocampal and cerebral cortical slices in vitro (Satoh et al., 1997; Maekawa et al., 2000). In rat thymocyte membranes, *S*-nitroso-L-cysteine inhibited adenylyl cyclase activity even after washing of the membranes with *S*-nitroso-L-cysteine-free buffer, but its inhibition was reversed by dithiothreitol treatment (Miyakoshi et al., 1998). We also reported that *S*-nitroso-L-cysteine regulated noradrenaline release (Naganuma et al., 1998) and Ca^{2+} mobilization from caffeine-sensitive intracellular Ca^{2+} pools (Naganuma et al., 1999) in PC12 cells (a neuronal cell line derived from rat pheochromocytoma). Recently, we reported that *S*-nitroso-L-cysteine inhibited [^{3}H]arachidonic acid release mediated by activation of cytosolic phospholipase A_2 with mastoparan (wasp venom peptide) in PC12 cells (Thang et al., 2000). In these studies, other synthetic nitric oxide compounds such as sodium nitroprusside, which stimulated cyclic GMP accumulation, did not elicit responses similar to those induced by *S*-nitroso-L-cysteine. These

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findings suggest that the biological and/or pharmacological effects induced by *S*-nitroso-L-cysteine are not due to nitric oxide and cyclic GMP, and suggest the possibility that *S*-nitroso-L-cysteine may be taken up in cells and slices without decomposition, and thus acts with the target proteins.

Interestingly, noradrenaline release induced by *S*-nitroso-L-cysteine was eliminated by selective amino acids such as L-leucine and L-phenylalanine which were incorporated via the Na^+-independent system L amino acid transporter, and by 2-aminobicyclo[2.2.1]heptane-2-carboxylate (BCH, an inhibitor of system L; Palacín et al., 1998) in rat brain slices (Satoh et al., 1997). Li et al. (2000) also confirmed that L-leucine, but not D-leucine, inhibited *S*-nitroso-L-cysteine-induced noradrenaline release and showed that the effect induced by *S*-nitroso-L-cysteine was more potent than that induced by the *S*-nitroso-D-cysteine in rat spinal cord synaptosomes. In addition, not only BCH and L-phenylalanine but also *S*-nitroso-L-cysteine inhibited L-[^{3}H]leucine uptake in brain slices, and thus we proposed that *S*-nitroso-L-cysteine is incorporated via amino acid transporter(s) in rat brain slices (Satoh et al., 1997). In these studies, however, the role of glial cells, such as astrocytes, on the actions of *S*-nitroso-L-cysteine was not excluded. We reported that the inhibition of arachidonic acid release induced by *S*-nitroso-L-cysteine was eliminated by selective amino acids such as L-leucine in PC12 cells (Nemoto et al., 2001), but the existence of amino acid transporter(s) that can transport *S*-nitroso-L-cysteine was not examined. In addition, further studies are required to confirm the involvement of amino acid transporter(s) in the Ca^{2+} mobilization induced by *S*-nitroso-L-cysteine in neuronal cells. Thus, we investigated the involvement of amino acid transporter(s) in the actions of *S*-nitroso-L-cysteine in PC12 cells in the present study.

Many studies have established the character of amino acid transporters, their molecular structures, tissue expression, transport characteristics including Na^+ dependency, and so on (Palacín et al., 1998; Verrey et al., 1999; Kanai et al., 2000). System L has been characterized at the molecular level in several species; system L consists of two subunits, one of which is the heavy chain of the cell-surface antigen 4F2 (4F2hc) and the other is the light chain of 4F2, LAT1 (Kanai et al., 1998; Mastroberardino et al., 1998; Nakamura et al., 1999; Prasad et al., 1999) or LAT2 (Pineda et al., 1999; Segawa et al., 1999; Rossier et al., 1999; Rajan et al., 2000). Although LAT1 or LAT2 is not capable of amino acid transport on its own, LAT1 and LAT2 are capable of transport activity characteristic of the system L as a heterodimer with 4F2hc. In addition, it was established that system L prefers neutral amino acids with branched or aromatic side chains such as L-leucine, L-phenylalanine, L-methionine and L-valine, versus L-alanine, and that transport activity was Na^+ independent and inhibited by BCH.

In the present study, we show the presence of a specific amino acid transporter which is pharmacologically system L and prefers neutral amino acids such as L-leucine and L-phenylalanine, that *S*-nitroso-L-cysteine, but not other nitric oxide compounds, inhibits L-[^{3}H]leucine uptake, and that mRNAs of system L exist in PC12 cells. Further, Ca^{2+} mobilization induced by *S*-nitroso-L-cysteine from intracellular Ca^{2+} pools was inhibited by selective amino acids such as L-leucine. These findings confirm that *S*-nitroso-L-cysteine is incorporated into PC12 cells via the system L amino acid transporters.

2. Materials and methods

2.1. Materials

L-[^{3}H]Leucine (5.66 TBq/mmol, 153 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). L- and D-Isoforms of amino acids, Fura 2-acetoxymethyl ester, sodium nitroprusside, and $NaNO_2$ were obtained from Wako (Osaka, Japan). BCH and α-(methylamino)isobutyric acid (MeAIB, an inhibitor of the system A amino acid transporter; Palacín et al., 1998) were obtained from Sigma (St. Louis, MO, USA). *S*-Nitroso-L-cysteine and (±)-*N*-[(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexen-1-yl]-3-pyridine carboxamide (NOR-4) were purchased from Dojindo Lab. (Kumamoto, Japan). 3-Morpholino sydnonimine–HCl and *S*-nitroso-*N*-acetylpenicillamine were purchased from BIOMOL (Plymouth Meeting, PA, USA) and RBI (Natick, MA, USA), respectively.

2.2. Measurement of L-[^{3}H]leucine uptake in PC12 cells

PC12 cells were cultured on collagen-coated dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum as described previously (Naganuma et al., 1998, 1999; Nemoto et al., 2001). The detached PC12 cells were washed by centrifugation (200 × *g*, 2 min) at 4 °C and resuspended in modified Tyrode HEPES buffer (137 mM NaCl, 5 mM KCl, 5 mM glucose, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES (pH 7.4)). To study the effect of Na^+ on L-[^{3}H]leucine uptake, a Na^+-free Tyrode buffer containing 137 mM choline chloride was used in some experiments. Cell suspensions (40–60 μg protein, 0.8–1.2 × 10^6 cells per tube) were incubated with the indicated supplements for 8 min at 37 °C. The total volume was 0.2 ml. The reaction was terminated by the addition of 2 ml of ice-cold buffer. Free and incorporated L-[^{3}H]leucine was separated by filtration through Whatman GF/C glass filters. The cells on the filters were allowed to dry and were solubilized in 5 ml of a scintillation cocktail containing 20% Triton X-100. Radioactivity was determined by liquid scintillation spectrometry. Non-specific uptake was less than 1% of the total uptake in the normal and low Na^+ buffer.

2.3. Detection of mRNAs of amino acid transporters in PC12 cells by reverse transcriptase-polymerase chain reaction (PCR) analysis

Total RNA was prepared from PC12 cells using TRIzol reagent (Sigma, 1 ml/100 mm-dish). Total RNA (1 μg) was reverse-transcribed in a mixture containing oligo(dT)12–18 primer and superscript reverse transcriptase (Gibco BRL, Grand Island, NY, USA). PCR was carried out in a 15-μl reaction volume containing 1.5 μl of a cDNA mixture, 1 × Expand high-fidelity buffer containing 1.5 mM $MgCl_2$, 200 μM each dNTP, 20 μM each primer, and 1 U of Taq DNA polymerase (Gibco BRL). The DNA was denatured for 10 min at 95 °C prior to each PCR cycle of 95 °C for 1 min, each annealing temperature (50–65 °C) for 1 min, 72 °C for 1 min, followed by 7 min at 72 °C before refrigeration. For PCR amplification, we used the primers for rat LAT1, LAT2 and 4F2hc as follows: LAT1 primer set (sense: 5′-TCA AGC TCT GGA TCG AGC TGC TC-3′, and antisense: 5′-TCC TGT AGG GGT TGA TCA TCT CC-3′, the estimated size of the transcript was 438 bp), LAT2 primer set (sense: 5′-GCC TGT GGT ATC ATT GTT GTA GG-3′, and antisense: 5′-AGT TGA CCC ATG TGA GCA GC-3′, size 397 bp) and 4F2hc primer set (sense: 5′-CGA AGT GGA CAT GAA AGA TGT G-3′ and antisense: 5′-AAA CTA GGC CCT TCA CCT TCA G-3′, size 456 bp), as described previously (Kido et al., 2001). The number of cycles selected for each primer pair was found to produce a linear relation between the input RNA and the resulting PCR products. The PCR products were analyzed by electrophoresis on 1.5% agarose gel and visualized under UV light with ethidium bromide.

2.4. Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in PC12 cells

Levels of $[Ca^{2+}]_i$ in PC12 cells were determined as described previously (Naganuma et al., 1999) with minor modifications. Briefly, PC12 cells on dishes were loaded with 2 μM Fura 2-acetoxymethyl ester for 30 min at 37 °C in normal Na^+ buffer. The PC12 cells were washed and detached from the dish under a gentle stream of buffer. Detached cells were washed by centrifugation (800 × *g*, 30 s) at 4 °C with the indicated normal Na^+ or low Na^+ buffer, and resuspended in the indicated buffer. In some experiments, $CaCl_2$ was omitted from the assay mixture. Fluorescence readings were taken with a Hitachi F-2500 spectrophotometer.

2.5. Statistics

Values are means ± S.E.M. of the indicated numbers of independent experiments performed in triplicate. In the case of multiple comparisons, the significance of differences was determined using one-way analysis of variance followed by the Bonferroni test. In some cases, analysis was performed by using the paired *t*-test. *P* values at <0.05 were considered significant.

3. Results

3.1. Inhibition of L-[^{3}H]leucine uptake by S-nitroso-L-cysteine and L-phenylalanine, but not by L-alanine, in PC12 cells

First, we investigated the effects of *S*-nitroso-L-cysteine, L-phenylalanine and L-alanine on L-[^{3}H]leucine uptake in PC12 cells in normal Na^+-containing buffer and in low Na^+-(137 mM choline chloride-containing) buffer for 8 min. L-[^{3}H]Leucine uptake in the low Na^+ buffer was similar to that in the normal Na^+ buffer (Fig. 1). The addition of *S*-nitroso-L-cysteine to the assay mixture inhibited L-[^{3}H]leucine uptake in a concentration-dependent manner; the estimated ED_{50} values of *S*-nitroso-L-cysteine were 82.5 ± 6.0 and 120.5 ± 27.8 μM ($n=3$) in the low Na^+ and normal Na^+ buffer, respectively. The addition of L-phenylalanine also inhibited L-[^{3}H]leucine uptake in a concentration-dependent manner both in the low Na^+ and in the normal Na^+ buffer, and the addition of 1 mM L-phenylalanine almost completely inhibited L-[^{3}H]leucine uptake. The inhibitory effect of L-alanine on L-[^{3}H]leucine uptake was not significant at 0.3 mM, although the addition of concentrations greater than 0.3 mM L-alanine slightly inhibited uptake in both buffers.

3.2. Effects of L- and D-isoforms of amino acids, inhibitors and alternative substrates of amino acid transports on L-[^{3}H]leucine uptake in PC12 cells

Table 1 shows the effects of several amino acids on L-[^{3}H]leucine uptake in PC12 cells. The addition of L-isomers of leucine, phenylalanine, cysteine and methionine at 1 mM significantly inhibited L-[^{3}H]leucine uptake. Neither L-serine nor L-alanine inhibited uptake, and the inhibitory effect of L-threonine was limited. The addition of 0.3 mM L-leucine almost completely inhibited L-[^{3}H]leucine uptake because of dilution (20 nM L-[^{3}H]leucine versus 0.3 mM non-radioactive L-leucine). In contrast, the D-isomer of leucine at 0.3 mM did not inhibit L-[^{3}H]leucine uptake. At 1 mM, D-isomers of leucine and methionine significantly inhibited L-[^{3}H]leucine uptake, although D-isomers of phenylalanine, cysteine and serine showed no effect. Similar results were obtained in the low Na^+ buffer (data not shown, $n=3$). Thus, inhibition of L-[^{3}H]leucine uptake by amino acids in PC12 cells appeared to show amino acid selectivity and stereo-specificity.

The effects of classical inhibitors of amino acid transport systems, BCH (for system L) and MeAIB (for system A), on L-[^{3}H]leucine uptake were investigated (Table 2). The addition of 1 mM BCH, but not MeAIB, significantly inhibited L-[^{3}H]leucine uptake in PC12 cells in the low

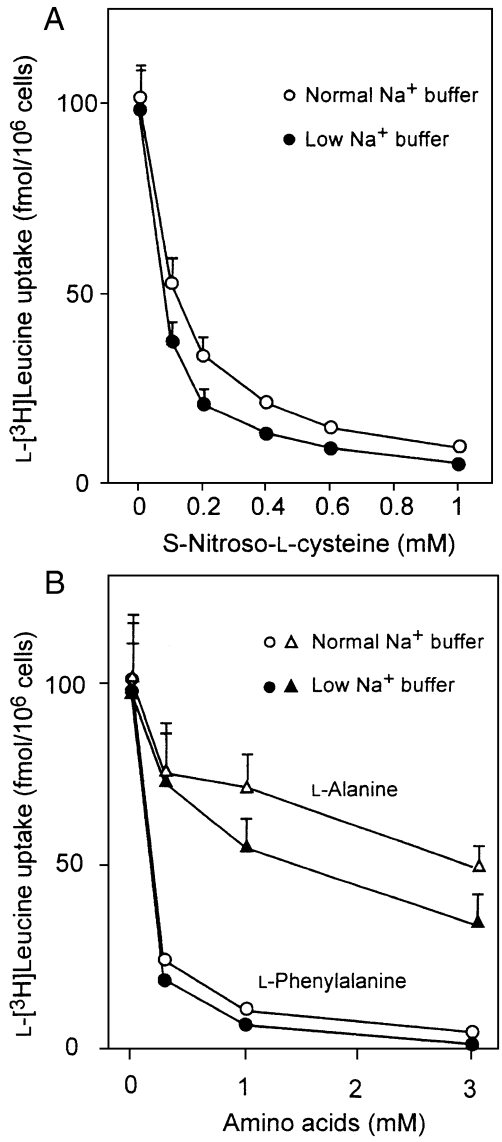

Fig. 1. Inhibition of L-[^{3}H]leucine uptake by *S*-nitroso-L-cysteine, L-phenylalanine and L-alanine in PC12 cells. PC12 cells were incubated with 20 nM L-[^{3}H]leucine for 8 min in normal Na^+ buffer (○△) and in low Na^+ buffer (●▲). The assay mixture was further supplemented with the indicated concentrations of *S*-nitroso-L-cysteine (○●) in Panel A, and L-phenylalanine (○●) and L-alanine (△▲) in Panel B. Values are the means ± S.E.M. for three independent experiments performed in triplicate.

and in the normal Na^+ buffer. Alternative substrates for H^+/monocarboxylate transporters, lactate and pyruvate (Halestrap and Price, 1999), at 1 mM did not inhibit L-[^{3}H]leucine uptake in PC12 cells in the low and the normal Na^+ buffer.

3.3. Effects of various nitric oxide compounds on L-[^{3}H]leucine uptake in PC12 cells

It has been shown that nitric oxide compounds regulate the release of neurotransmitters (Pogun and Kuhar, 1994; Lonart and Johnson, 1994,1995; Kaye et al., 1997). *S*-Nitrosothiols and *S*-nitroso-L-cysteine inhibited noradrenaline uptake in PC12 cells (Kaye et al., 1997) and in rat brain slices (Maekawa et al., 2001). Thus, we investigated the effects of various nitric oxide compounds, including *S*-nitroso-L-cysteine, on L-[^{3}H]leucine uptake in PC12 cells in the normal Na^+ buffer (Table 3). The addition of 1 mM sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine and $NaNO_2$, which at 0.5 mM stimulated cyclic GMP accumulation in PC12 cells (Naganuma et al., 1998), did not inhibit L-[^{3}H]leucine uptake in PC12 cells. NOR-4 (1 mM), which specifically releases nitric oxide radicals in neutral buffer (Kita et al., 1995), had no effect. 3-Morpholino syndonimine (1 mM), which yields peroxynitrite (Hogg et al., 1992) and does not stimulate cyclic GMP accumulation in PC12 cells (Naganuma et al., 1998), had no effect on L-[^{3}H]leucine uptake. In PC12 cells, *S*-nitroso-L-cysteine stimulated cyclic GMP accumulation at lower concentrations to about

Table 1
Effects of L- and D-isomers of various amino acids on L-[^{3}H]leucine uptake in PC12 cells

Addition	L-[^{3}H]Leucine uptake (fmol/10^6 cells)	
	L-Isomer	D-Isomer
None	119.8 ± 7.9	
Leucine (0.3 mM)	4.7 ± 2.1[a]	100.9 ± 10.2
Leucine (1 mM)	0.5 ± 0.5[a]	65.1 ± 10.5[a]
Phenylalanine (1 mM)	24.7 ± 4.9[a]	85.1 ± 8.1
Methionine (1 mM)	50.9 ± 7.1[a]	74.4 ± 0.7[a]
Cysteine (1 mM)	56.4 ± 15.5[a]	86.2 ± 19.6
Alanine (1 mM)	71.1 ± 11.2	Not determined
Serine (1 mM)	87.3 ± 10.0	106.6 ± 9.1
Threonine (1 mM)	72.8 ± 8.7	Not determined

PC12 cells were incubated with 20 nM L-[^{3}H]leucine for 8 min in normal Na^+ buffer. The assay mixture was further supplemented with the L- and D-isomers of the indicated amino acids. Values are the means ± S.E.M. for three or four independent experiments performed in triplicate. [a]$P<0.05$, statistically significant compared with the value without amino acids. Similar results were obtained in the low Na^+ buffer.

Table 2
Effects of BCH, MeAIB, lactate and pyruvate on L-[^{3}H]leucine uptake in PC12 cells

Addition	L-[^{3}H]Leucine uptake (%)	
	Low Na^+ buffer	Normal Na^+ buffer
None	100	100
BCH	15.3; 5.8	27.3 ± 7.5[a]
MeAIB	121.3; 77.3	103.2 ± 5.6
Lactate	106.4; 92.7	89.4; 109.2
Pyruvate	95.4; 87.0	81.8; 104.8

PC12 cells were incubated with 20 nM L-[^{3}H]leucine for 8 min in normal Na^+ buffer. The assay mixture was further supplemented with vehicle (None) or the indicated agents at 1 mM. Values are normalized as percentages of L-[^{3}H]leucine uptake in control PC12 cells, to reduce variation among experiments. The absolute values (fmol/10^6 cells) for L-[^{3}H]leucine uptake in low and normal Na^+ buffer were similar to those shown in Fig. 1. Some values are the results of two independent experiments performed in triplicate. Values in the normal Na^+ buffer are the means ± S.E.M. for three independent experiments. [a]$P<0.05$, statistically significant compared with the value without amino acids.

Table 3
Effects of various nitric oxide compounds on L-[^{3}H]leucine uptake in PC12 cells

Addition	L-[^{3}H]Leucine uptake (fmol/10^6 cells)
None	107.3 ± 1.7
SNC	23.0 ± 2.8[a]
SNP	107.5 ± 10.7
SNAP	112.4 ± 8.9
NOR-4	110.2 ± 10.0
SIN-1	105.2 ± 9.6
$NaNO_2$	114.9 ± 6.3

PC12 cells were incubated with 20 nM L-[^{3}H]leucine for 8 min in normal Na^+ buffer. The assay mixture was further supplemented with the indicated nitric oxide compounds at 1 mM. SNC, *S*-nitroso-L-cysteine. SNP, sodium nitroprusside. SNAP, *S*-nitroso-*N*-acetylpenicillamine. NOR-4, (±)-*N*-[(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexen-1-yl]-3-pyridine carboxamide. SIN-1, 3-morpholino sydnonimine. Values are the means ± S.E.M. for three independent experiments performed in triplicate. [a]$P < 0.05$, statistically significant compared with the value without nitric oxide compounds. Similar results were obtained in the low Na^+ buffer.

30 μM, but at higher concentrations over 30 μM it inhibited cyclic GMP accumulation, while the cyclic GMP level after stimulation with 0.3 mM *S*-nitroso-L-cysteine was 10-fold higher than the basal level (Naganuma et al., 1998). As shown in Fig. 1, *S*-nitroso-L-cysteine inhibited L-[^{3}H]leucine uptake at concentrations of 0.1 mM and higher in a concentration-dependent manner in the low and in the normal Na^+ buffer.

Previously, we reported that several responses induced by *S*-nitroso-L-cysteine in PC12 cells (Naganuma et al., 1998, 1999; Thang et al., 2000) and in rat thymocytes (Miyakoshi et al., 1998) were irreversible, not reversed by washing the cells and membranes with the *S*-nitroso-L-cysteine-free buffer. However, the inhibitory effect of *S*-nitroso-L-cysteine on L-[^{3}H]leucine uptake in the low Na^+ buffer was reversible and eliminated by washing PC12 cells with the *S*-nitroso-L-cysteine-free buffer; the L-[^{3}H]leucine uptake in PC12 cells preincubated with 0.6 mM *S*-nitroso-L-cysteine for 8 min and washed by centrifugation with the *S*-nitroso-L-cysteine-free buffer was 88.7 ± 5.7 fmol/10^6 cells, which was similar to that in the control cells (88.6 ± 9.4 fmol/10^6 cells).

3.4. Detection of LAT1, LAT2 and 4F2hc transcripts in PC12 cells by reverse transcriptase-PCR

The expression of the system L amino acid transporter in PC12 cells was examined by reverse transcriptase-PCR method using specific primers based on the respective nucleotide sequences of LAT1, LAT2 and 4F2hc (Fig. 2). The transcripts of LAT1 and LAT2 mRNAs (Panel A) and the transcript of 4F2hc mRNA (Panel B) were detected in the RNA from PC12 cells. The intensities of the 4F2hc (Panel B) and the LAT1 and LAT2 bands (data not shown) increased dependently on the PCR cycle number. The bands were not detected in samples without the reverse transcriptase treatment.

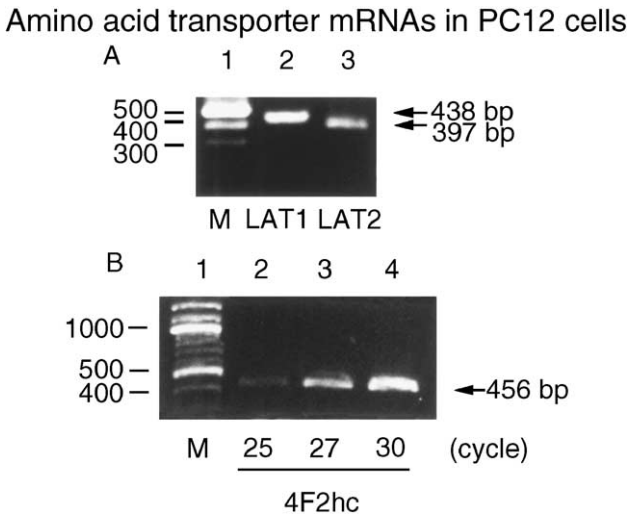

Fig. 2. Detection of mRNAs of amino acid transporters by reverse transcriptase-PCR in PC12 cells. Total RNA was isolated from PC12 cells and run in the presence of reverse transcriptase. In Panel A, the mRNA levels were analyzed by PCR (27 cycles) using the primers for LAT1 (lane 2) and LAT2 (lane 3), respectively. In Panel B, the primer for 4F2hc was used and the mixture was amplified by 25 (lane 2), 27 (lane 3) and 30 (lane 4) PCR cycles. In both panels, lane 1 shows 100-bp DNA ladder; 300–500 bp in Panel A and 400–1200 bp in Panel B. These are typical results from three independent experiments.

3.5. Effect of L-leucine on S-nitroso-L-cysteine-stimulated $[Ca^{2+}]_i$ increase from intracellular Ca^{2+} pools in PC12 cells

Previously, we reported that *S*-nitroso-L-cysteine stimulated $[Ca^{2+}]_i$ and that Ca^{2+} came from caffeine-sensitive intracellular Ca^{2+} pools in PC12 cells (Naganuma et al.,

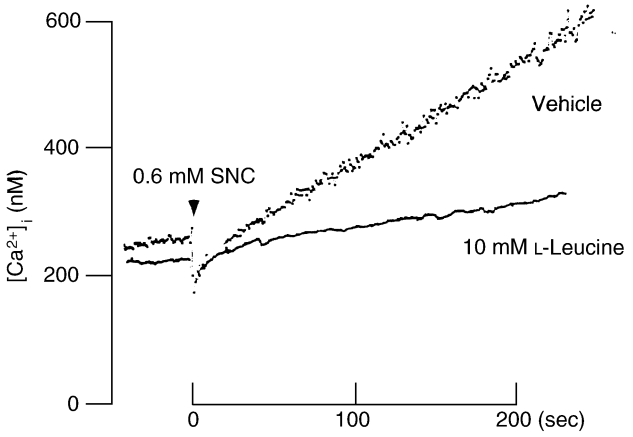

Fig. 3. *S*-Nitroso-L-cysteine-induced increase in $[Ca^{2+}]_i$ from intracellular Ca^{2+} pools and its inhibition by L-leucine in PC12 cells. Fura 2-acetoxymethyl ester-loaded PC12 cells were suspended in low Na^+ buffer. The cells were stimulated with 0.6 mM *S*-nitroso-L-cysteine (SNC) in the absence of extracellular $CaCl_2$. The assay mixture was further supplemented with vehicle or 10 mM L-leucine. These are typical results from three independent experiments. The initial drop in $[Ca^{2+}]_i$ was due to the effect of methanol in the *S*-nitroso-L-cysteine solution.

1999). We investigated the effects of *S*-nitroso-L-cysteine and L-leucine on $[Ca^{2+}]_i$ levels in PC12 cells in the low Na^+ buffer (Fig. 3). In the absence of extracellular $CaCl_2$, *S*-nitroso-L-cysteine at 0.6 mM caused a continuous increase in $[Ca^{2+}]_i$ in PC12 cells. The net increase in $[Ca^{2+}]_i$ produced by *S*-nitroso-L-cysteine in the low Na^+ buffer was similar to that in the normal Na^+ buffer (Naganuma et al., 1999). The addition of 10 mM L-leucine inhibited the increase in $[Ca^{2+}]_i$ induced by 0.6 mM *S*-nitroso-L-cysteine. The net increase in $[Ca^{2+}]_i$ induced by 0.6 mM *S*-nitroso-L-cysteine in the presence of 10 mM L-leucine was 85.4 ± 14.8 nM ($n = 3$), which was significantly lower ($P < 0.05$) than the net increase (328.2 ± 28.7 nM) in the absence of L-leucine. Similar results were obtained in the normal Na^+ buffer, and the addition of 10 mM BCH also inhibited the net increase in $[Ca^{2+}]_i$ induced by *S*-nitroso-L-cysteine in PC12 cells (data not shown, $n = 3$). The addition of 1 and 3 mM L-leucine or 10 mM L-alanine did not inhibit the effect of *S*-nitroso-L-cysteine.

4. Discussion

4.1. L-[^{3}H]leucine uptake via the system L, but not the system y^+L, amino acid transporter in PC12 cells

In the present study, we revealed the existence of transcripts for LAT1, LAT2 and 4F2hc in PC12 cells by reverse transcriptase-PCR analysis (Fig. 2). The mRNAs of LAT1 and LAT2 have been shown to be expressed at significant levels in the rat brain (Kanai et al., 1998; Nakamura et al., 1999; Pineda et al., 1999; Rossier et al., 1999). Since L-[^{3}H]leucine uptake was Na^+ independent and inhibited by 1 mM BCH in the present study, L-[^{3}H]leucine uptake in PC12 cells appeared to be mediated at least in part by system L. In addition, L-serine and L-threonine did not inhibit L-[^{3}H]leucine uptake in PC12 cells (Table 1). These two amino acids were good substrates for system L with LAT2 (Pineda et al., 1999; Segawa et al., 1999; Rossier et al., 1999; Rajan et al., 2000), and are poor substrates for system L with LAT1 (Kanai et al., 1998). The addition of 1 mM D-leucine and D-methionine significantly inhibited L-[^{3}H]leucine uptake in PC12 cells, although it was reported that system L with LAT2 did not interact with D-amino acids to a significant extent, but system L with LAT1 interacted with some D-amino acids with high affinity (Kanai et al., 1998; Rajan et al., 2000). Although we cannot exclude the involvement of system L with LAT2, these characteristics of L-[^{3}H]leucine uptake in PC12 cells are more compatible with those of 4F2hc/LAT1 than with those of 4F2hc/LAT2.

It has been shown that system y^+L, which consists of 4F2hc and y^+LAT1 or y^+LAT2, can transport cationic amino acids and neutral amino acid such as L-leucine, and that RNA of y^+LAT2 has a wider tissue distribution, including the brain, in humans and rats (Torrents et al., 1998; Pfeiffer et al., 1999; Bröer et al., 2000; Kanai et al., 2000). In *Xenopus* oocytes expressing system y^+L (y^+LAT1/4F2hc), L-phenylalanine and L-methionine were not taken up in the absence of Na^+, and L-leucine was taken up in the presence of Na^+ significantly more than that in the absence of Na^+ (Torrents et al., 1998; Pfeiffer et al., 1999). Kanai et al. (2000) also reported that Na^+ was required for L-[^{14}C]leucine uptake in CHO cells expressing system y^+L (y^+LAT1/4F2hc). In y^+LAT2/4F2hc-expressing oocytes, L-[^{14}C]leucine uptake was completely dependent on Na^+, and BCH and L-phenylalanine did not inhibit the uptake (Bröer et al., 2000). Although we did not examine the existence of system y^+L in PC12 cells, the sensitivity to Na^+, amino acids (L-phenylalanine and L-methionone) and BCH of L-leucine uptake by the system y^+L amino acid transporters was different from the present results obtained with PC12 cells.

4.2. Involvement of other amino acid transporter systems in L-[^{3}H]leucine uptake in PC12 cells

4F2hc is a common subunit associated with the second subunit with transporter activity, such as LAT1, LAT2, y^+LAT1, y^+LAT2 and cystine/glutamate transporter (named xCT) (Palacín et al., 1998; Verrey et al., 1999; Kanai et al., 2000). Although the system X_c^- amino acid transporter (xCT/4F2hc) is a Na^+-independent transporter and is expressed in the brain, system X_c^- could not transport L-leucine and the activity was not inhibited by BCH (Sato et al., 1999). System $b^{o,+}$, which consists of rBAT (related to $b^{o,+}$ amino acid transporter) and BAT1 ($b^{o,+}$ amino acid transporter), exhibited activity as a Na^+ independent transporter of L-cystine as well as basic and neutral amino acids such as L-leucine (Chairoungdua et al., 1999). However, the activity of system $b^{o,+}$ was much higher in the small intestine and kidney but quite low in the brain (Palacín et al., 1998; Verrey et al., 1999), and mRNA of BAT1 was not expressed in the rat brain (Chairoungdua et al., 1999). Recently, Kim et al. (2001) identified T-type amino acid transporter 1 (TAT1), which exhibits Na^+-independent transport of aromatic amino acids including L-phenylalanine, but not L-leucine, and which bears similarity (about 30% identity) to protein sequences of mammalian proton-linked monocarboxylate transporters. However, BCH did not inhibit TAT1-mediated amino acid uptake in their experiment. Although a family of proton-linked monocarboxylate transporters are able to transport monocarboxylates such as lactate and pyruvate (Halestrap and Price, 1999), 1 mM lactate and pyruvate did not inhibit L-[^{3}H]leucine uptake in PC12 cells. System A (designated ATA1-3) is expressed in various tissues, including the brain, and mediates the transport of neutral amino acids including L-glutamine, L-serine and MeAIB in a Na^+-coupled manner (Varoqui et al., 2000; Sugawara et al., 2000; Hatanaka et al., 2001). However, L-[^{3}H]leucine uptake was not dependent on

Na^+, and both L-serine and MeAIB did not inhibit L-[^{3}H]leucine uptake in PC12 cells. These findings suggest that L-[^{3}H]leucine uptake in PC12 cells was not mediated by system X_c^-, system $b^{o,+}$, T-type transporter, H^+/monocarboxylate transporters, or system A.

4.3. *S*-nitroso-L-cysteine uptake via the system L amino acid transporter in PC12 cells

Previously, we showed that L-[^{3}H]leucine uptake was inhibited by *S*-nitroso-L-cysteine in rat brain slices (Satoh et al., 1997). In the present study, we revealed that *S*-nitroso-L-cysteine inhibited Na^+-independent and BCH-sensitive L-[^{3}H]leucine uptake in PC12 cells. *S*-Nitroso-L-cysteine inhibited L-[^{3}H]leucine uptake in micromolar concentrations (ED_{50} value was about 100 μM) like L-phenylalanine. In addition, the effects of *S*-nitroso-L-cysteine on $[Ca^{2+}]_i$ mobilization (Fig. 3) and on arachidonic acid release (Nemoto et al., 2001) were inhibited by L-leucine and BCH in PC12 cells. L-[^{3}H]Leucine uptake was inhibited by *S*-nitroso-L-cysteine, but not by other synthetic nitric oxide compounds such as sodium nitroprusside (Table 3). These findings suggest that nitric oxide radicals, peroxynitrite, or cyclic GMP do not mediate the inhibitory effect of *S*-nitroso-L-cysteine on L-[^{3}H]leucine uptake in PC12 cells. Although we reported many pharmacological effects induced by *S*-nitroso-L-cysteine in various cell types, as described in Section 1, these cellular responses were irreversible and thus appeared to be mediated by *S*-nitrosylation of the target proteins (Miyakoshi et al., 1998; Naganuma et al., 1999; Thang et al., 2000). However, the inhibitory effect of *S*-nitroso-L-cysteine on L-[^{3}H]leucine uptake was completely eliminated by washing with *S*-nitroso-L-cysteine-free buffer, which is probably not due to covalent modifications such as *S*-nitrosylation of amino acid transporter(s). To our knowledge, this is the first study to show that system L, probably the 4F2hc/LAT1 complex, participates in the transport of the endogenous nitric oxide molecule, *S*-nitroso-L-cysteine, into neuronal cells. Davisson et al. (1996) suggested that stereo-selective recognition sites of *S*-nitroso-L-cysteine may exist on vascular smooth muscles, since the L-isoform of *S*-nitroso-cysteine produced a more pronounced vasodilation than did the D-isoform. Boulos et al. (2000) suggested that peroxynitrite ($ONOO^-$) was transported into the cytosol through the ion channel (HCO_3^-/Cl^- anion exchanger) in human platelets. Some nitric oxide-related molecules including *S*-nitroso-L-cysteine appear to be taken up in cells by transporters and/or binding sites.

There remain several questions to be answered. In PC12 cells, neither L-valine nor L-isoleucine, which are good substrates for system L, inhibited the effect of *S*-nitroso-L-cysteine on arachidonic acid release (Nemoto et al., 2001). In rat brain slices, L-arginine, a poor substrate for system L, inhibited the response induced by *S*-nitroso-L-cysteine, and L-histidine, a good substrate for system L, showed no effect in Na^+-free buffer (Satoh et al., 1997). It is reported that different cells contain a distinct set of amino acid transport systems in their plasma membranes, as combination of ubiquitous (systems A, L, y^+L, etc.) and tissue-specific transport systems (system $b^{o,+}$, etc.), and that the transport systems show overlapping specificities (Palacín et al., 1998). *S*-Nitroso-L-cysteine may be taken up by other types of amino acid transporter and/or by unidentified transporters, in addition to system L. Molecular identification with antisense oligonucleotides of the transporters and/or mutant cells lacking the respective transporters will be an important next step.

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