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Multiple pathways of σ_1 receptor ligand uptakes into primary cultured neuronal cells

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Abstract

Although many antipsychotics have affinities for σ receptors, the transportation pathway of exogenous σ_1 receptor ligands to intracellular type-1 σ receptors are not fully understood. In this study, σ_1 receptor ligand uptakes were studied using primary cultured neuronal cells. [^{3}H](+)-pentazocine and [^{3}H](*R*)-(+)-1-(4-chlorophenyl)-3-[4-(2-methoxyethyl)piperazin-1-yl]methyl-2-pyrrolidinone L-tartrate (MS-377), used as a selective σ_1 receptor ligands, were taken up in a time-, energy- and temperature-dependent manner, suggesting that active transport mechanisms were involved in their uptakes. σ_1 receptor ligands taken up into primary cultured neuronal cells were not restricted to agonists, but also concerned antagonists. The uptakes of these ligands were mainly Na^+-independent. Kinetic analysis of [^{3}H](+)-pentazocine and [^{3}H]MS-377 uptake showed K_m values (μM) of 0.27 and 0.32, and V_{max} values (pmol/mg protein/min) of 17.4 and 9.4, respectively. Although both ligands were incorporated, the pharmacological properties of these two ligands were different. Uptake of [^{3}H](+)-pentazocine was inhibited in the range 0.4–7.1 μM by all the σ_1 receptor ligands used, including *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine monohydrochloride (NE-100), a selective σ_1 receptor ligand. In contrast, the inhibition of [^{3}H]MS-377 uptake was potently inhibited by haloperidol, characterized by supersensitivity (IC_{50}, approximately 2 nM) and was inhibited by NE-100 with low sensitivity (IC_{50}, 4.5 μM). Moreover, kinetic analysis revealed that NE-100 inhibited [^{3}H]MS-377 uptake in a noncompetitive manner, suggesting that NE-100 acted at a site different from the uptake sites of [^{3}H]MS-377. These findings suggest that there are at least two uptake pathways for σ_1 receptor ligands in primary cultured neuronal cells (i.e. a haloperidol-sensitive pathway and another, unclear, pathway). In addition, pretreatment of cells with a calmodulin antagonist, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7), a myosin light chain kinase inhibitor, 1-(5-chloronaphthalene-1-sulfonyl)homopiperazine (ML-9), or microsomal Ca^{2+}-ATPase inhibitors resulted in a reduction of the amount of σ receptor ligand uptake. These findings suggest that the Ca^{2+} pump on the endoplasmic reticulum and/or calmodulin-related events might be involved in the regulation of the uptake of σ receptor ligands into primary neuronal cells.

Keywords: Haloperidol; MS-377; NE-100; Uptake; Antipsychotic; σ_1 receptor

1. Introduction

The existence of σ receptors was initially postulated based on the psychotomimetic effects of a benzomorphan, *N*-allylnormetazocine ((+)-SKF-10,047) (Martin et al., 1976). Many antipsychotics, antidepressants and immunosuppressants have some affinity for σ receptors (Walker et al., 1990). In addition, several lines of evidence indicate that σ receptors in the central nervous system are involved in drug addiction and affective disorders (Weissman et al., 1991; Witkin, 1994), and those in the immune system are involved in immunoregulation (Liu et al., 1995). Subsequent biochemical and pharmacological studies using radioligands have demonstrated that the σ receptors are distinct from opioid or phencyclidine (PCP) binding sites, and

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therefore can be classified into at least two subtypes designated as "σ_1" and "σ_2". Recently, cDNAs of σ_1 receptors were cloned, and their amino acid sequences were deduced (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1997, 1998; Prasad et al., 1998). We also reported that amino acid substitutions (Ser99Ala, Tyr-103Phe and di-Leu105,106di-Ala) in the transmembrane domain of σ_1 receptors play a critical role in ligand binding (Yamamoto et al., 1999).

The σ_1 receptor is a ubiquitous protein and the primary structure is highly conserved among a variety of mammalian species and tissues, indicating its importance in cellular functions. Subcellular σ_1 receptors are mainly distributed in the microsomal and nuclear fractions (McCann et al., 1994; Hanner et al., 1996; Alonso et al., 2000). Immunostaining by Western blot analysis using membrane fractionation revealed that σ_1 receptors are mainly present in microsomal fractions, but are also present in mitochondrial fractions and, to a certain extent, in synaptosomal fractions (Yamamoto et al., 1999; Hayashi et al., 2000). Recently, Alonso et al. (2000) reported that σ_1 receptor immunostaining is associated with the plasma membrane and with the membrane of some mitochondria. These findings suggest that σ_1 receptors exist mainly in intracellular organelles rather than in the plasma membrane and, therefore, σ receptor ligands react with σ_1 receptors inside the cells. If this is true, it should be of interest to find how exogenously added σ receptor ligands access the intracellular σ_1 receptor, for example, the σ_1 receptor in the mitochondria or the endoplasmic reticulum. Although many drugs are reported to have affinities for σ_1 receptors, the system by which σ receptor ligands are taken up by brain cells remains unknown. In the present study, we explored the possibility that σ receptor ligands could be incorporated into cells through multiple pathways, and we characterized substrate specificity and mechanisms of uptake using radiolabeled σ_1 receptor ligands.

2. Materials and methods

2.1. Materials

MS-377 ((*R*)-(+)-1-(4-chlorophenyl)-3-[4-(2-methoxyethyl)piperazin-1-yl]methyl-2-pyrrolidinone L-tartrate) and [^{3}H]MS-377 (specific activity, 1,180 GBq/mmol) were synthesized by Mitsui Chemicals, *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine monohydrochloride (NE-100) was synthesized (Okuyama et al., 1993) in the Department of Organic Chemistry at the Research Center, Taisho Pharmaceutical (Saitama, Japan). [^{3}H](+)-pentazocine (specific activity, 1,036 GBq/mmol) was purchased from New England Nuclear. 2,5-Di-(*t*-butyl)-1,4-hydroquinone (BHQ) and thapsigargin were purchased from Calbiochem (La Jolla, CA). *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) was obtained from Research Biochemicals (Natick, MA). All other chemical reagents were of analytical grade. Pregnant Wistar–Imamichi rats were purchased from Imamichi Institute for Animal Reproduction (Ibaraki, Japan).

2.2. Cell culture

Primary neuronal cell cultures were prepared by mechanoenzymatic dissociation of the fetal rat telencephalon, according to a modification of the method described by Yuzaki et al. (1990). In brief, the rat embryos were removed at 17 gestational days, under ether anesthesia, and the telencephalon, including the cortex, hippocampus and striatum, was dissected out and freed from the meninges. Following treatment with trypsin (0.25%, Difco) in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (CMF-PBS) containing 0.02% DNAase I (Sigma) for 10–15 min at 37 °C on the shaker, the enzyme solution was removed by aspiration. To inactivate the residual enzyme, a mixture of equal parts of Dulbecco's modified Eagle's medium (DMEM) and heat-inactivated horse serum was added to the cells. The cells were mechanically dispersed by repetitive pipetting and filtered through a nylon mesh to remove undissociated cells. The cells were rinsed three times with culture medium and plated on a polyethyleneimine (Sigma)-coated well (0.32 cm^2) at a final density of $0.5–1.5 \times 10^6$ cells per ml. The culture was maintained for 7 days with defined culture medium: DMEM supplemented with 1 mg/ml bovine serum albumin, 10 μg/ml insulin, 1 nM 3,3′,5-triiodo-L-thyronine, 0.1 mg/ml human transferrin, 1 μg/ml aprotinin, 100 μM putrescine, 1 mM sodium pyruvate, 10 nM progesterone, 30 nM selenium, in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.3. σ receptor ligand uptake

After removal of the defined culture medium, the cells were washed twice with warm DMEM. After the addition of 65 μl of DMEM, the cells were preincubated for 30 min with or without the drugs in a humidified atmosphere of 5% CO_2 in air at 37 °C.

The standard uptake study was started by the addition of 25 μl of [^{3}H]MS-377 (14 nM) or [^{3}H](+)-pentazocine (70 nM) in DMEM in a humidified atmosphere of 5% CO_2 in air at 37 °C (total volume; 100 μl), and was terminated by rapid aspiration of the media, followed by washing eight times with ice-cold CMF-PBS and twice with an acidic high-sodium solution (0.5 M NaCl solution adjusted to pH 2.5 with HCl) according to the method of Haigler et al. (1980). Incubation time for the standard uptake study was 20 min while for the kinetic study the incubation time was 4 min. The cells were solubilized by the addition of 50 μl of 10% SDS. Radioactivity was measured by liquid scintillation spectrophotometry (Packard; Packard, Meriden, CT) in 2 ml of Pico-Fluor (NEN, Boston, MA, USA) at a counting efficiency of 45–50%. Nonspecific binding

was determined by the addition of haloperidol (10 μM) for [^{3}H](+)-pentazocine or haloperidol (3 μM) for [^{3}H]MS-377 binding studies, respectively. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, USA).

To prepare Na^+-free uptake solution, sodium chloride in the HEPES buffered solution (140 mM NaCl, 5 mM HEPES, 5 mM KCl, 1 mM KH_2PO_4, 1.8 mM $CaCl_2$, 0.4 mM $MgSO_4$, 5 mM glucose, pH 7.6) was replaced by choline chloride. To prepare Ca^{2+}-free uptake solution, the $CaCl_2$ in the above solution was replaced with 1 mM EGTA.

The transport kinetic parameters (K_m and V_{max}) were determined using a Lineweaver–Burk plot. Values are expressed as the means ± S.E.M. Measurements were compared between experimental groups using one-way analysis of variance with pairwise comparison by the Bonferroni method.

3. Results

3.1. Time course and Na^+ dependence of σ receptor ligand uptake into primary cultured neuronal cells

As shown in Fig. 1A, the selective σ_1 receptor ligand, [^{3}H](+)-pentazocine, was taken up by the primary cultured neuronal cells. To measure the ligand uptake into the cells, the cells incubated at 37 °C were washed with acidic high-sodium solution (pH 2.5) to wash out surface-bound radioactivity (acid-wash). After acid washing, 77–86% of the radioactivity remained in the cells compared with the cells after PBS washing (Fig. 1A). Similarly, a newly developed, selective σ_1 receptor ligand, [^{3}H]MS-377, was also taken up into the neuronal cells (Fig. 1B). The uptake of these two ligands was a linear function of time for 4 min (data not shown, $n = 2$). When performed at 4 °C, cellular uptakes were lower by > 88% than that obtained at 37 °C for both ligands, indicating that most of the uptakes are temperature-dependent (Table 1). Moreover, sodium azide, a metabolic inhibitor, significantly inhibited the uptake of [^{3}H](+)-pentazocine or [^{3}H]MS-377 (in the presence of 10 mM NaN_3, 69.5 ± 1.7% of control (5)* or 82.7 ± 10.8 (5); 30 mM NaN_3, 41.5 ± 2.5 (5)* or 65.6 ± 6.3 (5)*, respectively; * $p < 0.05$).

Table 1
Temperature-dependent accumulation of σ receptor ligands in neuronal cultured cells

Temperature (°C)	[^{3}H](+)-pentazocine (DPM/μg protein/20 min)	[^{3}H]MS-377
4	60.9 ± 11.1	32.5 ± 0.6
37	541.2 ± 12.5[a]	343.5 ± 6.8[a]

Each value represents the mean ± S.E.M. of four determinations.
[a] $P < 0.0001$.

We further investigated whether the uptake of σ receptor ligands was Na^+-dependent. The Na^+ dependence of the uptake process was tested by disrupting the Na^+ gradient with ouabain (50 μM), an inhibitor of Na^+, K^+-ATPase. Ouabain (50 μM) failed to influence [^{3}H](+)-pentazocine or [^{3}H]MS-377 uptake, 92.3 ± 4.2% of the control (5) or 96.8 ± 3.0 (4), respectively. We also examined the effect of removing extracellular Na^+ by replacing NaCl equi-osmotically with choline chloride. Removal of the Na^+ ions not only failed to inhibit uptake, but instead increased the amounts of σ receptor ligands taken up (Fig. 2). These findings indicated that the major component of the uptakes was independent of extracellular Na^+ ions.

The uptake process was studied using an endocytosis inhibitor, phenylarsine oxide. The uptakes of the two

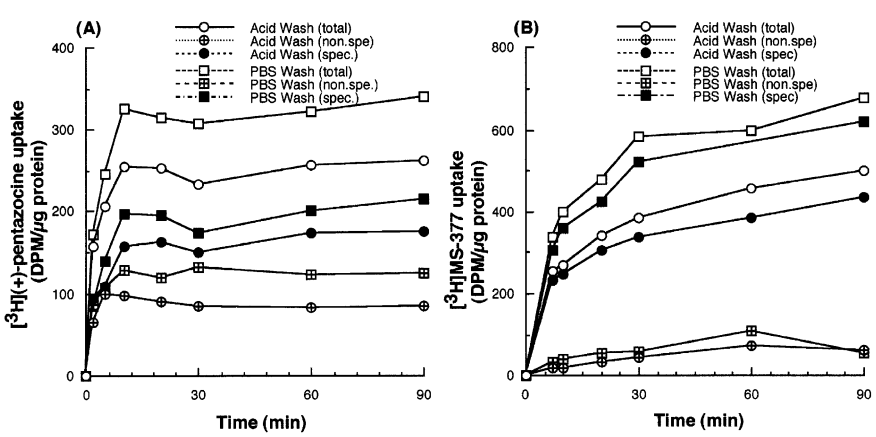

Fig. 1. Time-dependent uptake of [^{3}H](+)-pentazocine (A) and [^{3}H]MS-377. The primary cultured neuronal cells were incubated at 37 °C in DMEM. Nonspecific binding was determined in the presence of 10 μM (A) or 3 μM (B) (+)-pentazocine. After incubation, the cells were washed with CMF-PBS eight times, and subsequently with acidic high-sodium solution twice (Acid Wash), or washed with only CMF-PBS eight times (PBS Wash). Each point represents the mean of four determinations.

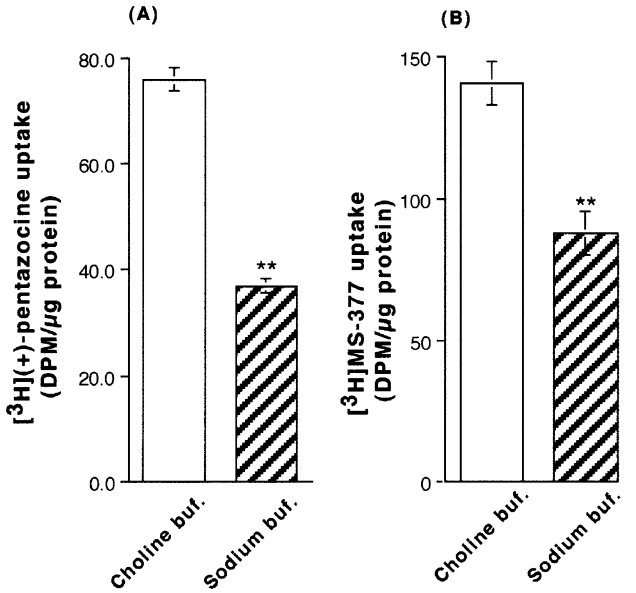

Fig. 2. The specific uptake of [^{3}H](+)-pentazocine (A) and of [^{3}H]MS-377 (B) in the presence (Sodium buf.) or absence (Choline buf.) of Na^+ in the HEPES buffer. Choline buffer was made by replacing NaCl osmotically with choline chloride in the HEPES buffer. The values are the means ± S.E.M. from 6 to 10 determinations. $^*p < 0.01$.

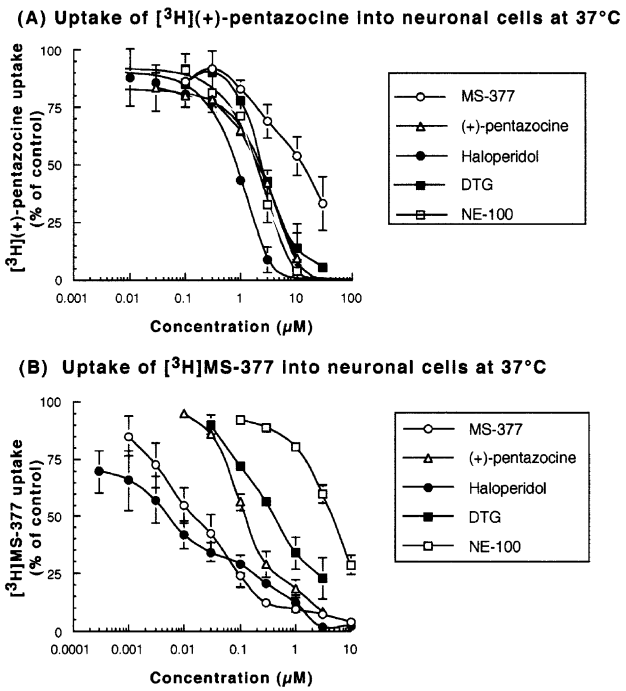

Fig. 3. Inhibition of specific [^{3}H](+)-pentazocine uptake (A) or [^{3}H]MS-377 uptake (B) into the neuronal cultured cells by σ receptor ligands. The cells were preincubated with σ receptor ligands for 30 min in DMEM, and then incubated with [^{3}H]MS-377 or [^{3}H](+)-pentazocine at 37 °C for 20 min. The uptake measured in the absence of inhibitors was taken as 100%, and values are the means ± S.E.M. for three to eight independent experiments. In the upper graph, the Hill coefficient for MS-377 is 0.41 and the other is 0.63–1.16. In the lower graph, the Hill coefficient for haloperidol and MS-377 is 0.35 and 0.55, respectively, and the other is 0.73–1.01.

radioligands, [^{3}H](+)-pentazocine and [^{3}H]MS-377, showed similar properties, while there was a difference in phenylarsine oxide sensitivity. The uptake of [^{3}H](+)-pentazocine was inhibited by treatment with 10 μM phenylarsine oxide (49.1 ± 1.0% of the control, $n = 4$), while the uptake of [^{3}H]MS-377 was less potently affected by treatment with phenylarsine oxide (72.6 ± 2.9% of the control, $n = 5$).

3.2. Substrate selectivity

As shown in Fig. 3, the inhibition curves of [^{3}H](+)-pentazocine uptake and [^{3}H]MS-377 uptake into neuronal cultured cells at 37 °C with σ receptor ligands were significantly different. The uptake of [^{3}H](+)-pentazocine was inhibited by σ receptor ligands in the following order of potency: haloperidol > NE-100 > (+)-pentazocine > DTG > MS-377 (Table 2). These IC_{50} values of the [^{3}H](+)pentazocine uptake by σ receptor ligands, except DTG, were concomitant with the reported K_i values in membrane binding of [^{3}H](+)-pentazocine (Takahashi et al., 1999, 2000) (Fig. 4A). However, uptake of [^{3}H]MS-377 was inhibited by these ligands in the following order: haloperidol > MS-377 > (+)-pentazocine > DTG > NE-100 (Table 2). The uptake of [^{3}H]MS-377 was quite sensitive to haloperidol and its inhibition curve of [^{3}H]MS-377 uptake was shallow, as shown in Fig. 3B (Hill coefficient, $n_H = 0.35$). In particular, the uptake of [^{3}H]MS-377 was less potently inhibited by NE-100 (Fig. 3B). As shown in Fig. 4B, these IC_{50} values of [^{3}H]MS-377 uptake by the σ receptor ligands tested, except NE-100, were relatively well correlated with its affinity for σ_1 receptors (K_i values in membrane binding of [^{3}H]MS-377 reported by Karasawa et al., 2000).

3.3. Kinetics of σ receptor ligands uptake

Concentration dependency was observed in both uptakes. The Lineweaver–Burk plots of uptakes of [^{3}H]MS-377 at 37 °C were linear while the plot of uptake of [^{3}H](+)-pentazocine was slightly curved (Fig. 5). Using [^{3}H](+)-pentazocine as a ligand, the K_m value was 0.27 ± 0.06 μM and the V_{max} was 17.4 ± 3.9 pmol/mg pro-

Table 2
Uptake at 37 °C into primary cultured neuronal cells

σ receptor ligands	IC_{50} (μM)			
	[^{3}H](+)-pentazocine	N	[^{3}H]MS-377	N
Haloperidol	0.43 ± 0.16	3	0.0015 ± 0.0006	4
MS-377	7.10 ± 2.26	3	0.023 ± 0.0088	5
NE-100	1.32 ± 0.62	3	4.54 ± 1.05	5
(+)-pentazocine	2.06 ± 0.27	4	0.18 ± 0.04	4
DTG	3.35 ± 0.93	3	0.50 ± 0.09	3

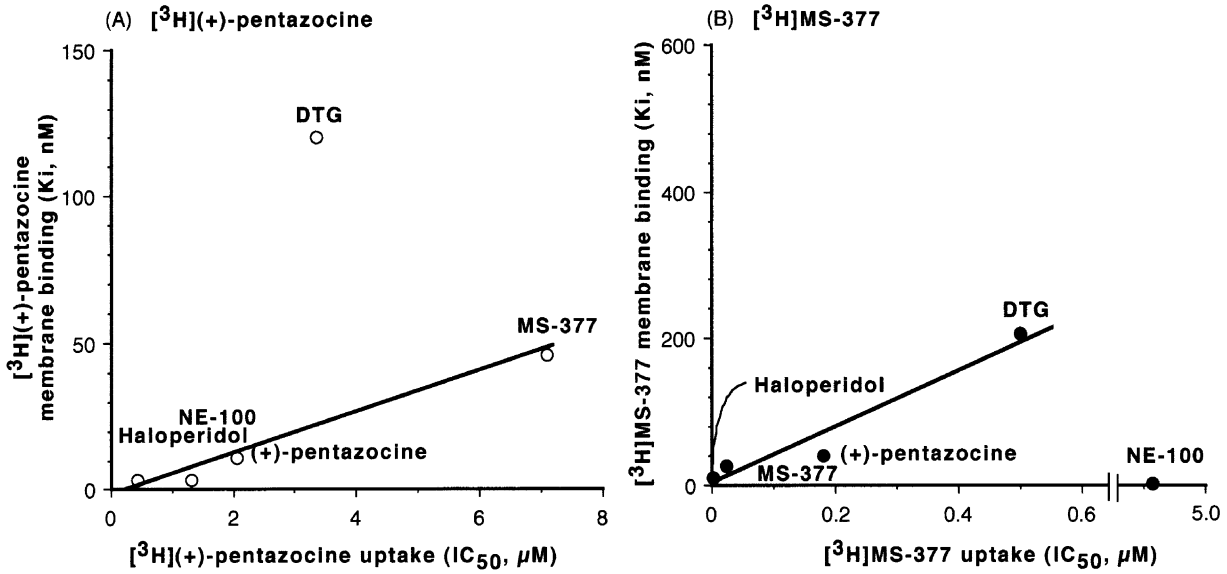

Fig. 4. The horizontal axis in A and in B represents the IC_{50} values obtained using [^{3}H](+)-pentazocine uptake and [^{3}H]MS-377 uptake into the cells, respectively. The vertical axis in A and in B represents the K_i values obtained with membrane binding using [^{3}H](+)-pentazocine and [^{3}H]MS-377, respectively. (A) Correlation between the reported K_i values (Takahashi et al., 1999, 2000) and the potency to inhibit [^{3}H](+)-pentazocine uptake into primary cultured neuronal cells. (B) Correlation between the reported K_i values (Karasawa et al., 2000) and the potency to inhibit [^{3}H](+)MS-377 uptake into primary cultured neuronal cells.

tein/min ($n = 5$). Using [^{3}H]MS-377 as a ligand, the K_m value was 0.32 ± 0.04 μM and the V_{max} value was 9.43 ± 1.43 pmol/mg protein/min ($n = 5$). The affinities (K_m) for the uptakes of [^{3}H](+)-pentazocine and [^{3}H]MS-377 were similar while there was a small enrichment of the maximum uptake rate (V_{max}) in [^{3}H](+)-pentazocine uptake (~1.8-fold) compared with [^{3}H]MS-377 uptake. The inhibition of [^{3}H](+)-pentazocine uptake by 3 μM MS-377 was in a competitive manner (Fig. 5A), while inhibition of [^{3}H]MS-377 uptake by 3 μM (+)-pentazocine showed a mixed mechanism (Fig. 5B). In contrast, inhibition of the uptake of [^{3}H]MS-377 by 3 μM NE-100 was in a noncompetitive manner (Fig. 5C).

3.4. Effect of intracellular Ca^{2+} or calmodulin on [^{3}H]MS-377 uptake

BHQ and thapsigargin are the most widely used membrane permeable selective inhibitors of the endoplasmic reticulum Ca^{2+} pump (Thastrup et al., 1990; Demaurex et al., 1992). In most cells, these drugs inhibit the pump and result in the passive leakage of Ca^{2+} from the inositol

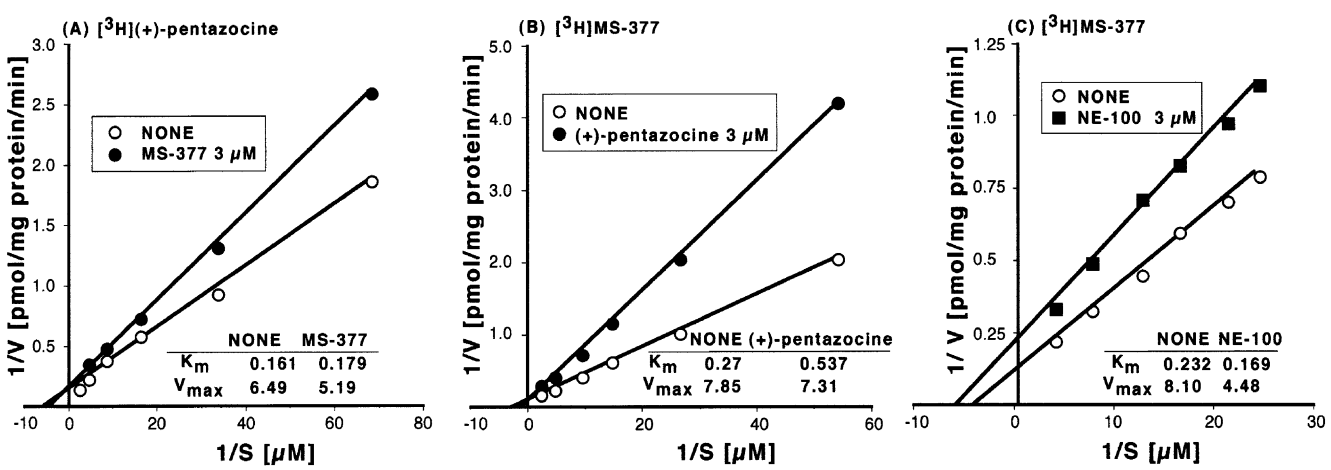

Fig. 5. The specific uptakes of [^{3}H](+)-pentazocine (A) and of [^{3}H]MS-377 (B,C). The cells were incubated for 4 min in DMEM. The measurements from typical experiments were transformed using a Lineweaver–Burk plot. Values are from a typical experiment. Nonspecific binding for (A) or (B,C) was determined in the presence of 10 or 3 μM haloperidol, respectively.

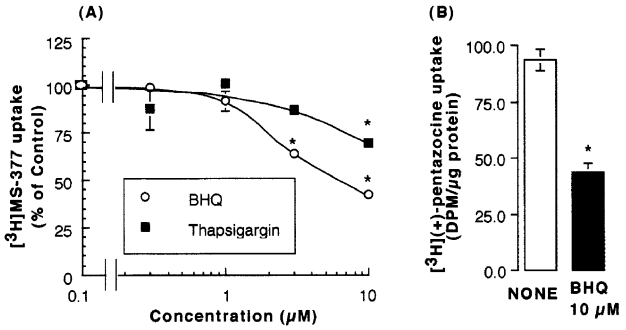

Fig. 6. Effect of BHQ or thapsigargin on the specific uptake of [^{3}H]MS-377 (A) and [^{3}H](+)-pentazocine (B). The values are the means ± S.E.M. from five determinations. $^*p < 0.05$.

1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} store leading to an increase in $[Ca^{2+}]i$ followed by capacitative Ca^{2+} entry. As shown in Fig. 6, the uptakes of [^{3}H](+)-pentazocine and [^{3}H]MS-377 at 37 °C were reduced by pretreatment with BHQ or thapsigargin. To clarify whether intra- or extracellular Ca^{2+} was involved in the modulation of uptakes, we carried out the uptake studies in the presence or absence of Ca^{2+} in combination with EGTA (1 mM) or a general Ca^{2+} blocker La^{3+} (100 μM). The presence or absence of extracellular Ca^{2+} had no effect on the BHQ-induced σ receptor ligand uptake reduction, while blockade of BHQ-induced Ca^{2+} entry by La^{3+} did not reverse but also enhanced the decrease in BHQ-induced σ receptor ligand uptake (Table 3). Using a calcium ionophore ionomycin, removal of intracellular Ca^{2+} by EGTA slightly reduced the uptakes of [^{3}H]MS-377, indicating that decreases in the intracellular Ca^{2+} concentrations had little effect on σ receptor ligand uptake.

Next, the effect of calmodulin on the uptake was examined. A calmodulin antagonist, W-7, potently inhibited the uptake of [^{3}H]MS-377 in the presence or absence of extracellular Ca^{2+} (Table 3). The IC_{50} of W-7 was 3.16 μM ($n = 4$) or 2.12 μM ($n = 2$) in [^{3}H]MS-377 or [^{3}H](+)-pentazocine uptake, respectively. Using another calmodulin antagonist calmidazolium, at the concentration of 10 μM, the uptake of [^{3}H](+)-pentazocine was more potently inhibited (57.4% of the control, $n = 3$) than the uptake of [^{3}H]MS-377 (73.1% of the control, $n = 4$). W-7 is also known to inhibit Ca^{2+}/calmodulin-dependent phosphodiesterase and myosin light chain kinase (MLCK) (Itoh and Hidaka, 1984; Hidaka et al., 1981). Therefore, we used a MLCK inhibitor, 1-(5-chloronaphthalene-1-sulfonyl)homopiperazine, ML-9. ML-9 potently blocked the uptake of [^{3}H](+)-pentazocine (IC_{50} 1.17 μM; $n = 2$) and the uptake of [^{3}H]MS-377 (IC_{50} 2.70 μM; $n = 3$).

4. Discussion

4.1. Multiple pathways of σ_1 receptor ligands uptake into the neuronal cells

In the present study, we investigated the uptake of exogenously added σ receptor ligands using primary cultured neuronal cells. We used two selective σ_1 receptor radioligands: [^{3}H](+)-pentazocine and [^{3}H]MS-377. In the present study, both ligands were taken up into cells. The uptake was temperature-, time-, and energy-dependent, suggesting that some active transport systems take part in the uptake of these two ligands. Interestingly, the uptakes of these two ligands appeared not to be mediated via the same pathway. A different pathway was also indicated by the different pharmacology of the inhibitors. A protein tyrosine phosphatase inhibitor, phenylarsine oxide, which is an endocytosis inhibitor, almost completely blocked the uptake of [^{3}H](+)-pentazocine but poorly blocked that of [^{3}H]MS-377. Phenylarsine oxide is a trivalent arsenical which can form relatively stable ring structures with vicinal sulfhydryl groups. The formation of these structures on

Table 3
Effect of Ca^{2+} on the uptake of [^{3}H](+)-pentazocine and [^{3}H]MS-377

	[^{3}H](+)-pentazocine (% of control)		[^{3}H]MS-377 (% of control)	
	Mean ± S.E.M	*N*	Mean ± S.E.M	*N*
Ca^{2+} 1.8 μM + BHQ 10 μM	59.0 ± 3.8	5	37.8 ± 1.3	4
EGTA + BHQ 10 μM	48.0 ± 2.2	5	41.9 ± 1.9	4
Ca^{2+} 1.8 μM + BHQ 10 μM + La^{3+} 100 μM	32.3 ± 3.4	5	26.0 ± 2.5	4
Ca^{2+} 1.8 μM + ionomycin 2 μM	65.7 ± 2.9	5	92.4 ± 1.2	5
EGTA + ionomycin 2 μM	66.5 ± 3.7	5	93.6 ± 4.5	5
Ca^{2+} 1.8 μM + W-7 10 μM	15.1 ± 1.6	5	32.2 ± 1.9	4
EGTA + W-7 10 μM	18.3 ± 3.4	5	29.7 ± 2.2	4
Ca^{2+} 1.8 μM + ML-9 1 μM	42.6 ± 2.4	5	67.8 ± 4.1	4
EGTA + ML-9 1 μM	42.5 ± 2.4	5	64.5 ± 0.2	4

Cells were not preincubated since cells were easily detached in the presence of EGTA. Therefore, cells were incubated with radiolabeled ligands at 37 °C for 10 min.

the plasma membrane inhibits receptor internalization (Hertel et al., 1985). Considering the finding that phenylarsine oxide could not block the uptake of [^{3}H]MS-377, the cellular uptake of [^{3}H]MS-377 is probably not mediated by receptor-mediated endocytosis. In relation to receptor-mediated endocytosis, it is unclear whether the σ_1 receptor exists on the cell surface membranes. Previously, we reported that the σ_1 receptor was detected in rat brain synaptosomal fractions, using an antiserum against σ_1 receptor fragment, while most large populations of σ_1 receptor immunoreactivities are in the microsomal fractions (Yamamoto et al., 1999). Therefore, a small but significant number of σ_1 receptors might exist on the cell surface. In a recent study of immunocytochemical localization of the σ_1 receptor in the adult rat central nervous system, Alonso et al. (2000) detected ultrastructural immunostaining patterns in portions of the membrane facing the synaptic contacts; labeling appeared to be accumulated within the postsynaptic membrane thickening. Thus, σ_1 receptors also exist in the proximal plasma membrane. Therefore, it would be important to determine to which subcellular site of the σ_1 receptors each σ receptor ligand binds.

By incubating at 4 °C, the uptake of [^{3}H]MS-377 was suppressed to a minimum and [^{3}H]MS-377 bound to the cell surface. Under such conditions, the IC_{50} values obtained by displacement with other σ receptor ligands (IC_{50} in μM at 4 °C, $n = 3$: Hal, 0.54; (+)-pentazocine, 8.21; MS-377, 2.75; NE-100, 7.08) were not proportional to the affinity for the σ_1 receptor obtained from membrane binding (Karasawa et al., 2000). However, [^{3}H]MS-377 uptake was highly sensitive to haloperidol at 37 °C. The IC_{50} value against haloperidol (approximately 2 nM) was close to the K_i values derived from membrane binding (10 nM, Karasawa et al., 2000). As shown in Fig. 4B, IC_{50} values with other σ receptor ligands, except NE-100, were proportional to the K_i values reported by Karasawa et al. (2000) ($r = 0.97$). In contrast, NE-100 could not inhibit intracellular [^{3}H]MS-377 uptake despite its high affinity for the σ_1 receptor. These findings suggest the possibility that subcellular storage of MS-377 may be in compartments distinct from that of NE-100.

Although IC_{50} values for [^{3}H](+)-pentazocine uptake by other σ receptor ligands were approximately two orders of magnitude less potent than the K_i values for membrane binding, the IC_{50} values obtained, except for DTG, were proportional to the K_i values reported by Takahashi et al. (1999, 2000) (in Fig. 6A, $r = 0.99$). Considering that DTG is not a selective σ_1 receptor ligand but is also a σ_2 receptor ligand, σ_2 receptor ligands might also be incorporated into the neuronal cells.

Together, these findings suggest that at least two σ_1 receptor ligand uptake pathways exist in neuronal cultured cells. One pathway appears to be a common pathway of σ receptor ligands including NE-100, which was inhibited not only by σ_1 receptor ligands but also by σ_2 receptor ligands. The other pathway was quite sensitive to haloperidol but insensitive to NE-100 (designated as the haloperidol-sensitive pathway). These findings are consistent with the analysis of [^{3}H]MS-377 showing that [^{3}H]MS-377 uptake was potently inhibited by haloperidol and noncompetitively by NE-100, suggesting that NE-100 may interact with sites different from the [^{3}H]MS-377 uptake site.

These multiple binding/storage sites inside the cells might be involved in different cellular signaling caused by various σ_1 receptor ligands. Considering that the uptake of MS-377 is very sensitive to haloperidol, it is of interest that MS-377 may be a possible antipsychotic candidate, and that the target site of MS-377 is located inside the neurons but is different from the NE-100 binding site. Recently, the existence of two subtypes of σ_1 receptors was hypothesized because at least two subtypes of σ_1 receptors may differentially affect glutamate NMDA neurotransmission in the terminal and origin regions of the mesolimbic and nigrostriatal dopaminergic systems (Gronier and Debonnel, 1999). It is unclear whether these subtypes of σ_1 receptors are directly related to multiple pathways of σ receptor ligand uptakes, although the present findings also demonstrated the existence of a functional multiplicity of σ_1 receptor ligands.

4.2. Modulation of σ_1 receptor ligand uptakes into the neuronal cells

We observed that the uptakes of both σ_1 receptor ligands, [^{3}H](+)-pentazocine and [^{3}H]MS-377, are likely to be dependent on calmodulin, since a calmodulin antagonist, W-7, potently reduced the uptake of these ligands. However, a Ca^{2+}/calmodulin kinase II inhibitor, KN-62, and a protein kinase C inhibitor, H-7, could not inhibit the uptake of σ_1 receptor ligands (data not shown). In addition, another calmodulin inhibitor, calmidazolium, inhibited the uptake of [^{3}H]MS-377 and [^{3}H](+)-pentazocine, suggesting that calmodulin itself was involved in the regulation of the σ_1 receptor ligand uptake process. The effects of calmidazolium are, at least in part, mediated by its calmodulin-antagonizing properties. That is, it acts by inducing Ca^{2+} release from filled storage compartments and its target of action is both the IP_3-sensitive store and the acidosome (Schlatterer and Schaloske, 1996). This suggests that Ca^{2+} release from the endoplasmic reticulum appears to be important for the regulation of uptake of σ receptor ligands.

However, another interesting approach is to use endoplasmic reticulum Ca^{2+} releasers. We found that inhibitors of the endoplasmic reticulum Ca^{2+} pump (BHQ or thapsigargin) inhibited the uptake of both [^{3}H](+)-pentazocine and [^{3}H]MS-377. Although these inhibitors are known to induce capacitative Ca^{2+} entry, removal of extracellular Ca^{2+} by EGTA, or block of capacitative Ca^{2+} entry by 100 μM of La^{3+} failed to restore the uptake of σ_1 receptor ligands. Taken together, Ca^{2+} release from endo-

plasmic reticulum and calmodulin function are probably involved in σ_1 receptor ligand uptakes; however, extracellular Ca^{2+} had no effect on it.

W-7 is known to inhibit Ca^{2+}/calmodulin-dependent phosphodiesterase and MLCK (Itoh and Hidaka, 1984; Hidaka et al., 1981). MLCK is widely distributed, not only in muscle cells, but also in nerve cells (Shimada et al., 1995). This protein kinase also phosphorylates the myosin light chain in a Ca^{2+}/calmodulin-dependent fashion (Hathaway et al., 1981). In the present study, an MLCK inhibitor, ML-9, almost completely blocked the uptake of both [^{3}H](+)-pentazocine and [^{3}H]MS-377. The effective concentration of ML-9 was most consistent with the inhibitory action on MLCK. Considering the evidence that vertebrate myosin I is involved in endocytosis (Raposo et al., 1999), the motor activity of myosin regulated by MLCK might be involved in σ_1 receptor ligand uptake in neuronal cultured cells.

The present study is, to our knowledge, the first report of σ_1 receptor ligand uptake in primary cultured neuronal cells. We have reported at least two different pathways for the uptake of σ_1 receptor ligand. These uptakes are probably regulated by calmodulin and/or Ca^{2+} release from intracellular Ca^{2+} stores but are not related to extracellular Ca^{2+}. Furthermore, these uptakes might be regulated by MLCK-mediated phosphorylation. Considering that many antipsychotics and antidepressants have affinities for σ receptor receptors, proper handling of these drugs requires that the mechanisms of accumulation/binding sites inside the neurons be clarified to reduce the side effects, or to control any additional effects of these drugs.

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