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Binding of σ receptor ligands and their effects on muscarine-induced Ca^{2+} changes in SH-SY5Y cells ☆

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Abstract

In human neuroblastoma SH-SY5Y cell preparations, σ_1 receptor agonists (+)-pentazocine and 1*S*,2*R*-(−)-*cis*-*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)cyclohexylamine (BD737) competed for [^{3}H]haloperidol binding with K_i values of 67 ± 10 and 14 ± 10 nM, respectively. (+)-Pentazocine or BD737 up to 10 μM did not affect basal levels of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in these cells, but they significantly reduced muscarine-induced $[Ca^{2+}]_i$ changes in a dose-related manner. However, the reduction by (+)-pentazocine was not reversed by the σ_1 receptor antagonist haloperidol. Further studies showed (+)-pentazocine, BD737 and haloperidol could compete for [^{3}H]quinuclidinyl benzilate binding in SH-SY5Y cells with K_i values of 0.51 ± 0.06, 0.32 ± 0.07 and 4.4 ± 2.3 μM, respectively. Thus, the inhibitory effects on muscarine-induced $[Ca^{2+}]_i$ changes by (+)-pentazocine and BD737 in SH-SY5Y cells were likely due to blockade of muscarinic receptors, not due to σ_1 receptor activation by these ligands.

Keywords: SH-SY5Y neuroblastoma; σ Receptor; Ca^{2+}, intracellular; Muscarine; (+)-Pentazocine; Haloperidol

1. Introduction

Since the original proposition of the σ receptor 25 years ago (Martin et al., 1976), the involvement of σ receptors in psychosis, cognition, neuroprotection, and locomotion in the central nervous system has been extensively examined in animal models. Sigma receptors, including both the σ_1 and σ_2 subtypes, were found to be widely distributed in the central nervous system and peripheral organs such as the adrenal gland, ovary, liver and kidney (Walker et al., 1990). They have also been shown to be present on a variety of human and rodent tumor cell lines (Vilner et al., 1995).

The signal transduction mechanisms of σ receptors have not been fully elucidated. A σ_1-binding protein of 223 amino acids was recently cloned from a guinea pig liver cDNA library (Hanner et al., 1996). This 25-kDa protein was found to be in endoplasmic reticular membranes and nuclear membranes, as well as plasma membranes (Dussossoy et al., 1999). It shares no homology with known neurotransmitter receptors or proteins involved in signal transduction. How this σ_1-binding protein is involved in the signal transduction of σ_1 receptors has yet to be fully unveiled. The σ_2 receptor has not been cloned. Recent advances have shown that both σ_1 and σ_2 receptors may affect intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) by mechanisms that are not yet clear. The σ_1 receptor agonist (+)-pentazocine at nanomolar concentrations was shown to potentiate bradykinin-induced $[Ca^{2+}]_i$ increase in NG-108 cells. This potentiation was blocked by σ_1 receptor antagonist *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride (NE-100) (Hayashi et al., 2000). Micromolar concentrations (10–100 μM) of 1*S*,2*R*-(−)-*cis*-*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)cyclohexylamine (BD737) and haloperidol, presumably acting on σ_2 receptors, produced a substantial transient $[Ca^{2+}]_i$ rise in human neuroblastoma SK-N-SH cells (Vilner and Bowen, 2000). In

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both cases, Ca^{2+} release from thapsigargin-sensitive intracellular Ca^{2+} stores was involved.

Previous work from our lab has examined the modulatory effects of σ_1 receptors on *N*-methyl-D-aspartate (NMDA)-stimulated neurotransmitter release from rat brain slices. The σ_1 receptor agonists (+)-pentazocine and BD737 were shown to inhibit NMDA-stimulated [^{3}H]dopamine release, whereas σ_1 receptor antagonists haloperidol and 1-(cyclopropylmethyl)-4-(2′ (4″-fluorophenyl)-2′ -oxoethyl)piperidine HBr (Dup734) were able to reverse the inhibition by (+)-pentazocine and BD737 (Gonzalez-Alvear and Werling, 1994). Since intracellular calcium plays an essential role in neurotransmitter release, the modulation of neurotransmitter release by σ_1 receptor ligands could possibly be a result from their effects on $[Ca^{2+}]_i$.

We have recently tested the effects of σ_1 receptor ligands on high K^+-stimulated dopamine release from human neuroblastoma SH-SY5Y cells (Ault and Werling, 2000). The SH-SY5Y cell line was subcloned from the SK-N-SH cells derived from a human sympathetic ganglion (Ross et al., 1983). Since SK-N-SH cells were shown to bear both σ_1 and σ_2 receptors (Vilner et al., 1995), it is likely that SH-SY5Y cells also have σ receptors. SH-SY5Y cells possess intracellular vesicles containing catecholamines. Various receptors (including muscarinic and bradykinin receptors) and ion channels (including L- and N-type Ca^{2+} channels) are present on SH-SY5Y cell membranes. Release of [^{3}H]-labeled catecholamines from SH-SY5Y cells has been linked to increase of $[Ca^{2+}]_i$ resulting from activation of muscarinic or bradykinin receptors or high K^+ stimulation (Vaughan et al., 1995). Mobilization of inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular Ca^{2+} stores was shown to be a major trigger for neurotransmitter release stimulated by both muscarinic and bradykinin receptor activation (Purkiss et al., 1995). Since SH-SY5Y cells have served a good model to study second messengers involved in neurotransmitter release (Vaughan et al., 1995), we chose this cell line as a model to examine possible effects of σ_1 receptor ligands on $[Ca^{2+}]_i$. In this study, we first characterized σ_1 receptors present in SH-SY5Y cells using radioligand binding assays, and then examined the effects of σ_1 receptor ligands on $[Ca^{2+}]_i$ changes induced following muscarinic receptor activation.

2. Methods

2.1. Chemicals

The following chemicals were purchased or obtained from these sources: [^{3}H]haloperidol (15 Ci/mmol) and [^{3}H]quinuclidinyl benzilate (Quinuclidinyl benzilate, L-[benzilic-4,4′ -^{3}H(N)]-) (42 Ci/mmol), New England Nuclear (Boston, MA, USA); atropine, (±)muscarine, haloperidol and spiroperidol hydrochloride, Research Biochemicals International (Natick, MA, USA); fura-2/AM (fura-2 acetoxymethyl ester), fluo-3/AM (fluo-3 acetoxymethyl ester), Pluronic F-127, Molecular Probes (Eugene, OR, USA); ionomycin, Calbiochem (La Jolla, CA, USA); Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, antibiotic–antimycotic 100× mix, Life Technologies (Gaithersburg, MD, USA); (+)-pentazocine, Research Technology Branch, National Institute on Drug Abuse (Rockville, MD, USA). BD737 was kindly provided by Dr. Wayen Bowen, National Institute of Digestive Disorders and Kidney (Bethesda, MD, USA). Endo-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-(1-methyl)ethyl-2-oxo-1*H*-benzamidazole-1-carboxamide hydrochloride (BIMU-8) was kindly provided by Dr. Douglas W. Bonhaus, Department of Neurosciences, Syntex Discovery Research (Palo Alto, CA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

2.2. Cell culture

SH-SY5Y cells (passage 26) were obtained from ATCC (Manassas, VA, USA). Cells were cultured in high-glucose DMEM with L-glutamine, sodium pyruvate and pyridoxine hydrochloride, supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 0.25 μg/ml amphotericin B in 5% CO_2/humidified air at 37 °C. Cells were passaged every 2 weeks in a splitting ratio of 1:20. Cells of passage 27–39 were used in experiments.

2.3. Radioligand binding studies

Assays using SH-SY5Y cell homogenates were done in Tris buffer (50 mM Tris–HCl, pH 7.4) with 10 mM $MgCl_2$. Methods of preparing SH-SY5Y cell membrane homogenates were adapted from McKenzie (1992). Briefly, confluent SH-SY5Y cells were harvested by trypsinization and washed twice with balanced salt solution–HEPES buffer (BSS–HEPES: NaCl 120 mM, KCl 5 mM, NaH_2PO_4 1.2 mM, $CaCl_2$ 1.5 mM, $MgCl_2$ 1.2 mM, dextrose 20 mM, HEPES 20 mM, pH 7.4). The cells were then resuspended in ice-cold Tris buffer with 10 mM $MgCl_2$, 1 mM EGTA, 5 μg/ml pepstatin, 10 μg/ml leupeptin, and 5 mM phenylmethylsulfonyl fluoride, and homogenized by a motor-driven glass-Teflon homogenizer for 15–20 strokes at about 2000 rpm. The homogenate was centrifuged at 1000 × *g* at 4 °C for 5 min. The supernatant was collected and centrifuged at 40,000 × *g* for 20 min. The pellets were resuspended in Tris buffer at a concentration of about 0.8 mg protein/ml, and stored at −80 °C until use. To label σ_1 receptor binding, a concentration of 4 nM [^{3}H]haloperidol was used in the presence of 100 nM spiroperidol to block dopamine D_2 and 5-HT_2 receptor binding. The σ_1 agonists (+)-pentazocine and BD737 were used to compete for σ_1 receptor binding. When (+)-pentazocine was used as a competing ligand, a 100 nM concentration of the σ_2 receptor-selective antagonist BIMU-8 was added to block σ_2 receptor binding. After a 2-h incubation at 25 °C, binding assays were terminated by addition of 3

ml ice-cold 10 mM Tris buffer (pH 7.4) and filtration through 0.1% polyethyleneimine (PEI)-soaked Whatman GF/B filters using a 48-well cell harvester (Brandel, Gaithersburg, MD, USA). Filters were then washed three times with 3 ml ice-cold Tris buffer, dried, and counted by liquid scintillation spectroscopy to measure radioactivity. Non-specific binding was measured in the presence of 1 μM haloperidol.

Whole-cell radioligand binding assays were done in BSS–HEPES buffer. Confluent SH-SY5Y cells were harvested by trypsinization, washed and resuspended in BSS–HEPES buffer. Competitive binding assays using [^{3}H]haloperidol to label σ_1 receptor binding were done similarly to that described above except that about 1.5 million cells were added into each binding reaction tube and incubated in BSS–HEPES buffer at 25 °C for 1.5 h.

In experiments measuring the affinities of σ_1 receptor ligands for muscarinic receptors, approximately 0.5 nM [^{3}H]quinuclidinyl benzilate was used to label muscarinic receptors. About 1.5 million cells were incubated in each binding reaction in BSS–HEPES buffer at 25 °C for 1.5 h, followed by filtration through 0.1% PEI-soaked Whatman GF/B filters and three 3-ml washes of ice-cold Tris buffer. Non-specific binding was measured in the presence of 1 μM atropine.

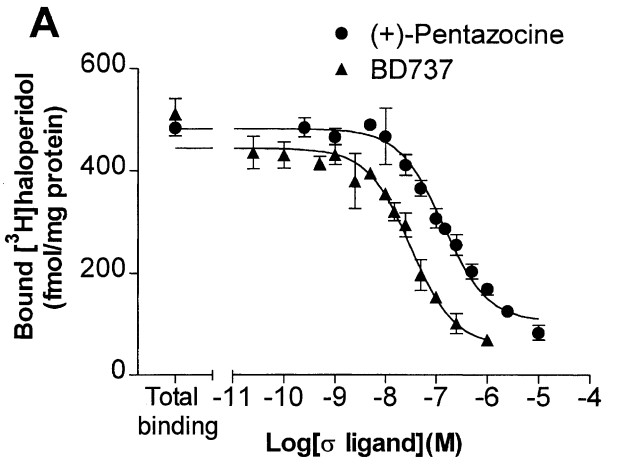
Data were analyzed using the MacLigand 4.97 (ABC Software, Baltimore, MD, USA) and Prism 3.0 programs (GraphPad Software, San Diego, CA, USA) to determine K_i and EC_{50} values. Protein concentrations of cell homogenates or suspensions were determined using a Bio-Rad DC protein kit (Bio-Rad Laboratories, Hercules, CA, USA). Bound radioactivity was converted to picomoles per milligram protein according to radioligand and protein concentrations.

2.4. $[Ca^{2+}]_i$ measurement in SH-SY5Y cell populations

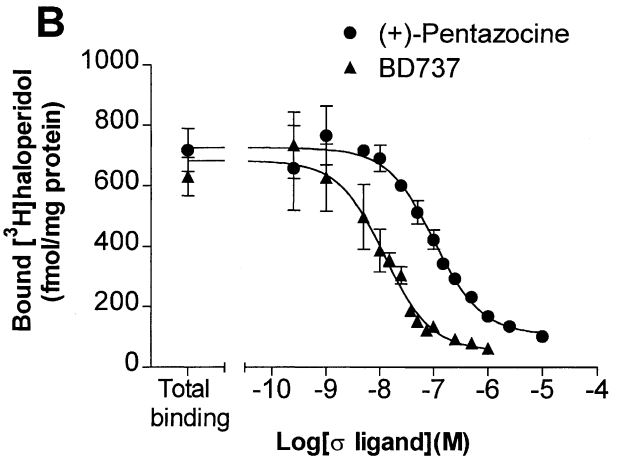
Confluent SH-SY5Y cells were harvested by trypsinization and washed twice with BSS–HEPES buffer. Cells were then resuspended at a density of 20 million/ml in BSS–HEPES containing 4 μM fura-2/AM and 0.02% Pluronic F-127, and incubated in the dark at room temperature for 1.5 h. After two washes of BSS–HEPES, cells were resuspended in BSS–HEPES in a density of ~5 million/ml. For each measurement, a 2-ml cell suspension was placed in a quartz cuvette. Dual-wavelength $[Ca^{2+}]_i$ measurement was done using a LS-50B spectrofluorimeter (Perkin-Elmer Analytical Instruments, Norwalk, CT, USA) at room temperature. Excitation wavelengths were 340 and 380 nm; emission was recorded at 509 nm. Calibration of $[Ca^{2+}]_i$ was done following the cell lysis method of Thomas and Delaville (1991) which uses digitonin to permeabilize cells. Autofluorescence was measured using cells not loaded with fura-2/AM.

2.5. $[Ca^{2+}]_i$ measurement in SH-SY5Y single cells

SH-SY5Y cells were seeded at a low density (~ 1×10^4 cells/ml) onto 15 mm diameter FisherBrand #1 microscope cover glasses coated with 2% gelatin and cultured till 80% confluency. Cover glasses with cells were incubated in BSS–HEPES buffer containing 3 μM fluo-3/AM or fura-



	K_i (nM)	
(+)-Pentazocine	67 ± 10	n = 3
BD737	14 ± 1.0	n = 4



	K_i (nM)	
(+)-Pentazocine	43 ± 7.0	n = 3
BD737	5.5 ± 1.3	n = 3

Fig. 1. Radioligand binding of σ_1 receptors in SH-SY5Y cells. Competition for [^{3}H]haloperidol by (+)-pentazocine and BD737 in SH-SY5Y whole-cell preparations (A) and SH-SY5Y cell membrane homogenates (B). [^{3}H]haloperidol (4 nM) was used to label σ_1 receptors in the presence of 100 nM spiroperidol to block dopamine D_2 and 5-HT_2 receptor binding, and a 100-nM concentration of the σ_2 receptor-selective antagonist BIMU-8 to block σ_2 receptor binding. Total binding was measured in the absence of BIMU-8. Non-specific binding measured in the presence of 1 μM non-labeled haloperidol was typically less than 15% of total binding in whole-cell preparations and less than 20% in cell homogenates. Shown were representative competition binding curves from a single experiment with triplicates (mean ± S.D.) analyzed with the GraphPad Prism 3.0 program. K_i values from pooled experiments of the indicated numbers (n) were determined with the MacLigand 4.97 program.

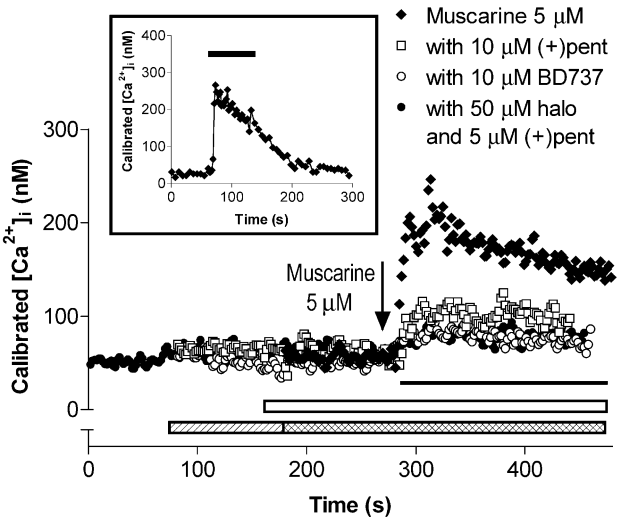


Fig. 2. Muscarine-induced $[Ca^{2+}]_i$ increase in SH-SY5Y cell suspensions and effects of (+)-pentazocine [(+)pent], BD737 or haloperidol. Three traces of calibrated $[Ca^{2+}]_i$ changes in approximately 1.0×10^7 SH-SY5Y cells loaded with fura-2/AM in 2 ml BSS–HEPES buffer were shown. The arrow and black bar marked the initiation and the duration of muscarine application, respectively. ♦ Muscarine alone; □ ○ application of 10 μM (+)-pentazocine or 10 μM BD737 is marked by the open bar; ● application of 50 μM haloperidol (halo) alone, and haloperidol with 5 μM (+)-pentazocine, was marked by the hatched and cross-hatched bars, respectively. Shown were representative traces from at least three experiments with similar responses. The inset showed a typical recording of $[Ca^{2+}]_i$ changes in a single SH-SY5Y cell exposed to a long stimulation by muscarine. The black bar marks the duration of 5 μM muscarine application.

2/AM and 0.025% Pluronic F-127 at 25 °C for 1.5 h, and washed twice with BSS–HEPES buffer. The cover glasses were then transferred to an RC-20 fast-flow chamber (Warner Instrument, Hamden, CT, USA), and placed on a Nikon Eclipse TE200 microscope (Nikon, Garden City, NJ, USA). Cells were perfused with BSS–HEPES buffer throughout experiments. Application of drugs in BSS–HEPES buffer was controlled by a VC-6 six-channel valve controller (Warner Instrument, Hamden, CT, USA).

For cells loaded with fluo-3/AM, the excitation wavelength was 488 nm. Emission images (512× 512 pixels, pixel size 0.351 μm) at 522 nm and transmitted light images were collected once per second with a Nikon Plan Apo × 60 oil-immersion objective (numerical aperture 1.40, iris 3.0 mm) by a Bio-Rad MRC 1024ES laser scanning confocal imaging system and analyzed with LaserSharp software (Bio-Rad Microscience, Herfordshire, UK). Fluorescent regions correlated with cells on transmitted light images were selected as regions of interest. Calibration of fluo-3 emission intensity ($[Ca^{2+}] = K_d \times (F - F_{min})/(F_{max} - F)$) was adapted from the method of Kao (1994), in which F_{max} and F_{min} were calculated from F_{Mn} (the fluorescence intensity when fluo-3 was completely saturated with Mn^{2+} (3 mM) after treating cells with 5 μM ionomycin). An average F_{Mn} value was obtained from eight calibration experiments, each with at least eight cells examined. Because $F_{Mn} = 0.2 \times F_{max} = 8 \times F_{min}$, the average F_{Mn} was used to calibrate $[Ca^{2+}]$. The K_d value of fluo-3 was 400 nM (Kao, 1994). Background fluorescence (measured after permeabilizing cells with digitonin) was subtracted before calibration.

For experiments with cells loaded with fura-2/AM, excitation lasers of 340 and 380 nm wavelength were controlled by a Lambda DG-4 Highspeed Filter Changer (Sutter Instrument, Novato, CA, USA). Fluorescent emission images (260 × 206 pixels) at 509 nm were collected with a Nikon Plan Fluor × 40 oil-immersion objective (numerical aperture 1.30) and a MicroMAX 5 MHz Digital Camera System (Princeton Instruments, Trenton, NJ, USA), and analyzed with the MetaFluor program (Universal Imaging, West Chester, PA, USA). Calibration of the ratio F_{340}/F_{380} to $[Ca^{2+}]$ was done using the equation $[Ca^{2+}] = K_d \times (R - R_{min})/(R_{max} - R) \times (S_{f,2}/S_{b,2})$ (Grynkiewicz et al., 1985). The K_d value of fura-2 was 224 nM. R_{min} and R_{max} values were determined under nominally 'zero Ca^{2+}' (0 Ca^{2+}, 10 mM EGTA, 5 μM ionomycin) and 'saturating Ca^{2+}' (10 mM Ca^{2+}, 5 μM ionomycin) conditions. $S_{f,2}/S_{b,2}$ was the ratio of F_{380} at 'zero Ca^{2+}' over F_{380} at 'saturating Ca^{2+}'. Background fluorescence of F_{380} and F_{340} measured with BSS–HEPES buffer present only was subtracted before calibration. Averages values of R_{max}, R_{min} and $S_{f,2}/S_{b,2}$ were obtained from eight calibration experiments, each with at least eight cells selected. All experiments measuring $[Ca^{2+}]$ in cell populations or single cells were carried out at room temperature.

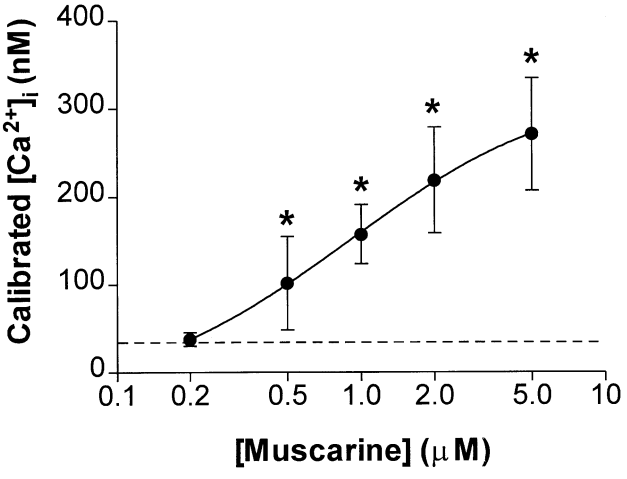


Fig. 3. Concentration response of muscarine-induced $[Ca^{2+}]_i$ changes in SH-SY5Y cells loaded with fura-2/AM. The peak values of muscarine-induced $[Ca^{2+}]_i$ increase were used for comparison. Data were expressed as mean values ± S.D. from 12 cells in four experiments. The dash line corresponded to the average basal $[Ca^{2+}]_i$ level. The solid curve was generated with non-linear regression analysis using GraphPad Prism 3.0 program. The computer-fitted EC_{50} value was 0.88 ± 0.22 μM. $^*P < 0.01$ compared with the basal level, one-way ANOVA followed by post hoc Dunnett's test.

3. Results

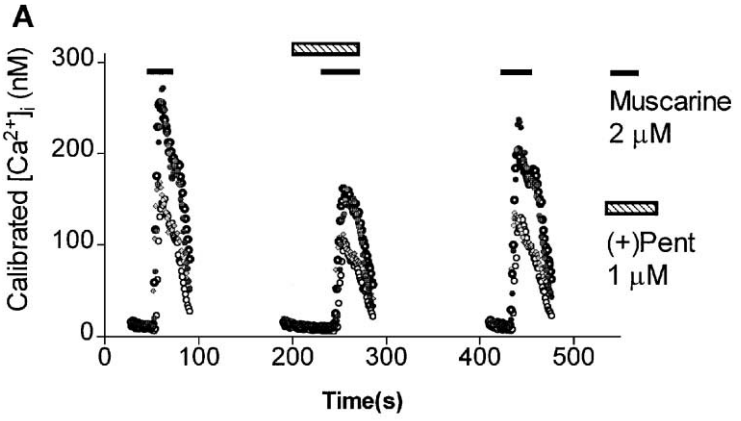
3.1. Radioligand binding of σ_1 receptors in SH-SY5Y cells

Binding of σ_1 receptors in SH-SY5Y cells was measured with 4 nM [^{3}H]haloperidol in the presence of 100 nM spiroperidol to block dopamine D_2 and 5-HT_2 receptors. The σ_1 receptor agonists (+)-pentazocine and BD737 were used to compete for [^{3}H]haloperidol binding. In whole-cell preparations in BSS–HEPES buffer (Fig. 1A), the K_i values for (+)-pentazocine and BD737 were 67 ± 10 and 14 ± 1.0 nM, respectively. In cell homogenates resuspended in Tris buffer (Fig. 1B), slightly lower K_i values for (+)-pentazocine (43 ± 7.0 nM) and BD737 (5.5 ± 1.3 nM) were obtained. In experiments in which (+)-pentazocine was used as a competing ligand, a 100 nM concentration of the σ_2 receptor-selective antagonist BIMU-8 (K_i for σ_2 receptor: 10 nM, Bonhaus et al., 1993) was used to block σ_2 receptor binding. Total binding measured without BIMU-8 present was not significantly larger than that measured with BIMU-8. Non-specific binding measured in the presence of 1 μM non-labeled haloperidol was typically less than 15% of total binding in whole-cell preparations and less than 20% in cell homogenates.

3.2. Muscarine-induced $[Ca^{2+}]_i$ changes in SH-SY5Y cells

Activation of muscarinic receptors by micromolar concentrations of muscarine induced a rapid $[Ca^{2+}]_i$ increase in SH-SY5Y cells. In cell suspension preparations, $[Ca^{2+}]_i$ usually rapidly rose to a peak level upon stimulation of muscarine, and gradually decreased in the presence of the persistent stimulation (Fig. 2). Exposure to micromolar concentrations of σ_1 receptor ligands BD737 (10 μM), (+)-pentazocine (10 μM), and haloperidol (50 μM) with (+)-pentazocine (5 μM) before application of muscarine was shown to substantially reduce the peaks of muscarine-induced $[Ca^{2+}]_i$ increase (Fig. 2). Similar inhibitory effects by (+)-pentazocine, BD737 and haloperidol were also observed when carbachol was used to activate muscarinic receptors and induce $[Ca^{2+}]_i$ responses.

In experiments measuring $[Ca^{2+}]_i$ changes in single cells, prolonged exposure to muscarine would usually produce a similar $[Ca^{2+}]_i$ response, that is, a fast rise and gradual decrease of $[Ca^{2+}]_i$ (Fig. 2, inset). In some cells, prolonged muscarine treatment induced oscillatory $[Ca^{2+}]_i$ changes with a decreasing magnitude (data not shown). In the following studies, the duration of muscarine application was controlled so as to induce only the initial fast $[Ca^{2+}]_i$ rising



B

Muscarine application sequence		Muscarine-induced $[Ca^{2+}]_i$ changes (% of 1st application of 2 μM muscarine)	
2nd	with pre-exposure of	(+)Pent 1 μM	(+)Pent 10 μM
		62 ± 4 % *†	20 ± 18 % *†
3rd	(+)Pent wash-out	85 ± 16 %	69 ± 25 % *

Fig. 4. $[Ca^{2+}]_i$ changes induced by 2 μM muscarine were reduced by pre-exposure to 1 or 10 μM (+)-pentazocine [(+)Pent]. (A) Representative responses of four cells loaded with fluo-3/AM. The black and hatched bars marked the application of 2 μM muscarine and 1 μM (+)-pentazocine, respectively. (B) Normalized results for muscarine-induced $[Ca^{2+}]_i$ changes (mean ± S.D.) in the presence of (+)-pentazocine [summarized data from 11 cells in two experiments for 1 μM (+)-pentazocine treatment and 18 cells in five experiments for 10 μM (+)-pentazocine treatment]. * Significantly different at $P<0.01$ compared with the first muscarine application by paired *t*-test, two-tailed; † significantly different at $P<0.01$ compared with the third muscarine application by paired *t*-test, two-tailed.

peaks. The peak values of $[Ca^{2+}]_i$ increase induced by muscarine were used for quantitative comparison. In SH-SY5Y cells, muscarine-induced $[Ca^{2+}]_i$ responses exhibited a dose relationship (Fig. 3). At concentrations between 500 nM and 5 μM, muscarine-induced $[Ca^{2+}]_i$ changes were significantly above the basal level. After stimulation of 1 and 2 μM muscarine, $[Ca^{2+}]_i$ rose to 0.16 ± 0.03 and 0.22 ± 0.06 μM, respectively. The EC_{50} value of muscarine from computer-assisted non-linear regression analysis was 0.88 ± 0.22 μM.

3.3. The effects of σ_1 receptor ligands on muscarine-induced $[Ca^{2+}]_i$ changes in SH-SY5Y single cells

This was tested using the following experimental paradigm: SH-SY5Y cells grown on cover glasses were loaded with fluo-3/AM or fura-2/AM, placed in a perfusion chamber on a microscope, and perfused with BSS–HEPES. Muscarine was applied three (Figs. 4 and 5) or four times (Fig. 6) during the experiment, with the first and last application being muscarine alone (duration 15–30 s), and the intermediate application(s) being muscarine (duration 20–40 s) with pre-exposure to σ_1 receptor ligands. The duration of muscarine application was controlled to induce just the initial $[Ca^{2+}]_i$ peaks. The duration of σ_1 receptor ligands pre-exposure was approximately 30 s. σ_1 Receptor drugs were also present during the intermediate muscarine application. Muscarine-induced $[Ca^{2+}]_i$ changes were defined as peak $[Ca^{2+}]_i$ values minus basal $[Ca^{2+}]_i$. For comparison, values of $[Ca^{2+}]_i$ response induced by subsequent muscarine application were normalized to the percentage of that induced by the first application in the same cells. $[Ca^{2+}]_i$ changes induced by muscarine with pre-exposure to σ_1 receptor ligands were compared with those induced by the first muscarine

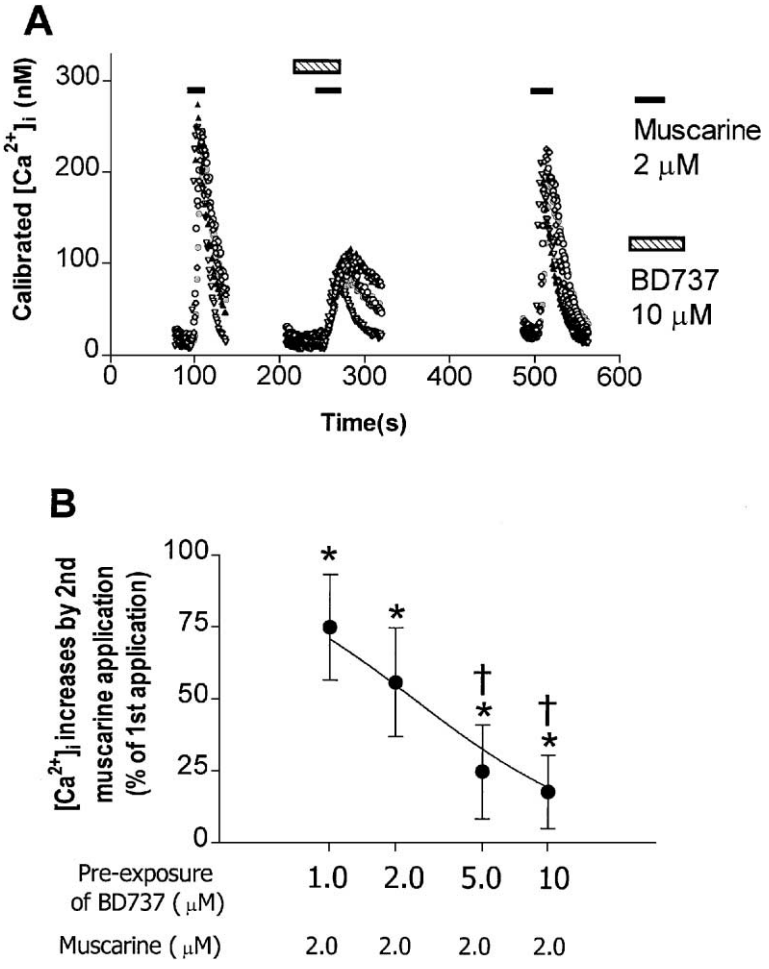


Fig. 5. $[Ca^{2+}]_i$ changes induced by 2 μM muscarine were reduced by pre-exposure to 1–10 μM BD737. (A) Representative responses of five cells loaded with fluo-3/AM. The black and hatched bars marked the application of 2 μM muscarine and 10 μM BD737, respectively. (B) Normalized results (percentage of $[Ca^{2+}]_i$ changes induced by first muscarine stimulation) of second muscarine-induced $[Ca^{2+}]_i$ changes (mean ± S.D.) with BD737 pre-exposure (summarized data from 20 cells in three experiments for 1 μM BD737, 28 cells in two experiments for 2 μM BD737, 25 cells in four experiments for 5 μM BD737, and 29 cells in four experiments for 10 μM BD737 treatment). The solid curve was generated by non-linear regression analysis using GraphPad Prism 3.0 program. The computer-fitted IC_{50} value was 2.4 ± 0.9 μM. * Significantly different at $P < 0.01$ compared with the first muscarine application by paired *t*-test, two-tailed; † significantly different at $P < 0.01$ compared with the third muscarine application by paired *t*-test, two-tailed.

application to examine effects of σ_1 receptor ligands; $[Ca^{2+}]_i$ changes induced by the last muscarine application were compared with those of the first application to examine the extent of muscarinic receptor desensitization.

In cells loaded with fluo-3/AM, prior exposure to micromolar concentrations of (+)-pentazocine significantly reduced the $[Ca^{2+}]_i$ increase induced by 2 μM muscarine. Representative responses of individual cells are shown in Fig. 4. Summarized results showed that 1 and 10 μM (+)-pentazocine significantly reduced muscarine-induced $[Ca^{2+}]_i$ changes to $61 \pm 4\%$ and $20 \pm 18\%$ of those of the first muscarine application, respectively. The reduced responses were also significantly different from those of the third application of muscarine alone ($85 \pm 16\%$ and $69 \pm 25\%$, respectively) after washing out (+)-pentazocine with BSS–HEPES ($P < 0.01$, paired *t*-test, two-tailed).

Micromolar concentrations of the σ_1 receptor ligand BD737 also significantly reduced muscarine-induced $[Ca^{2+}]_i$ changes in cells loaded with fluo-3/AM. Representative responses of individual cells are shown in Fig. 5A. Summarized results showed that pre-exposure to higher concentrations of BD737 conferred larger reduction of muscarine-induced $[Ca^{2+}]_i$ changes (Fig. 5B). The reduction caused by 1, 2, 5 and 10 μM BD737 was $75 \pm 8\%$, $56 \pm 9\%$, $25 \pm 6\%$ and $18 \pm 3\%$, respectively. The IC_{50} value from computer-assisted non-linear regression analysis was 2.4 ± 0.9 μM. The responses of the third muscarine application were approximately 70–85% of those of the first application, which were likely due to muscarinic receptor desensitization. However, the reduced $[Ca^{2+}]_i$ responses after pre-exposure to 5 and 10 μM BD737 were significantly different from those of the third muscarine application ($P < 0.01$, paired *t*-test, two-tailed), suggesting these reduced $[Ca^{2+}]_i$ changes were not primarily due to muscarinic receptor desensitization, but due to a direct inhibition by BD737.

To examine whether the reduction of muscarine-induced $[Ca^{2+}]_i$ changes was an effect mediated through σ_1 receptors, the σ_1 receptor antagonist haloperidol was tested to see

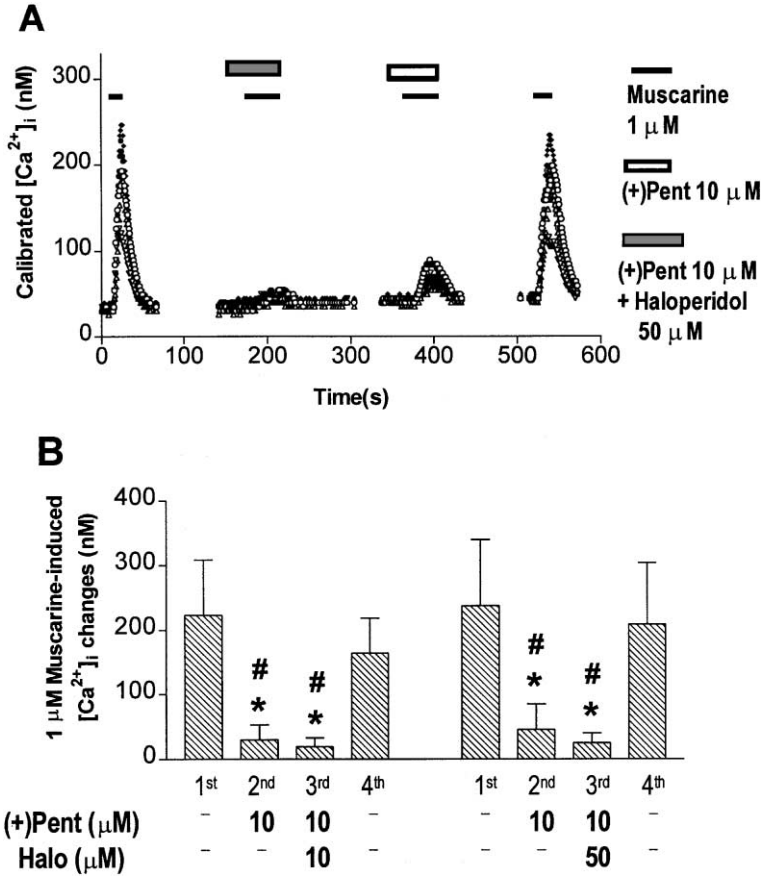
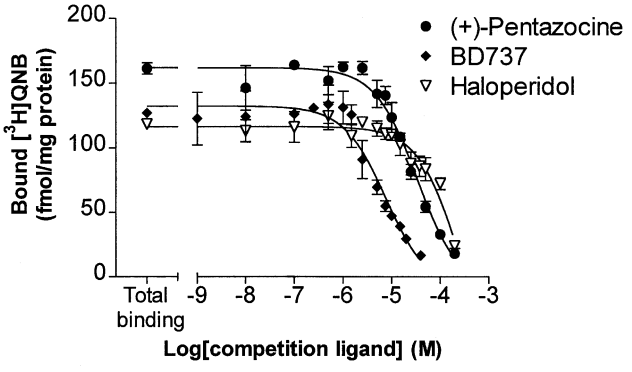


Fig. 6. The reduction of muscarine-induced $[Ca^{2+}]_i$ changes by (+)-pentazocine [(+)Pent] was not reversed by the σ_1 receptor antagonist haloperidol. (A) Representative responses of five cells loaded with fura-2/AM. The solid, open and hatched bar marked the application of 1 μM muscarine, 10 μM (+)-pentazocine, and 50 μM haloperidol with 10 μM (+)-pentazocine, respectively. (B) Muscarine (Musc)-induced $[Ca^{2+}]_i$ changes (mean ± S.D.) in the presence of (+)-pentazocine [(+)Pent] and haloperidol (Halo) (summarized data from 21 cells in four experiments with pre-exposure to 10 μM (+)-pentazocine and 10 μM haloperidol, and 25 cells in four experiments with pre-exposure to 10 μM (+)-pentazocine and 50 μM haloperidol. In some experiments, pre-exposure to 10 μM (+)-pentazocine preceded the third instead of the second application of 1 μM muscarine, as shown in A. * Significantly different at $P < 0.01$ compared with the first muscarine application by one-way ANOVA and post hoc Dunnett's test; # significantly different at $P < 0.01$ compared with the fourth muscarine application one-way ANOVA and post hoc Dunnett's test.

whether it could reverse the effects of σ_1 receptor agonists (+)-pentazocine and BD737. A similar experimental paradigm with four applications of muscarine was used. The first and fourth applications were muscarine alone, the second muscarine application was after a ~ 30-s exposure to (+)-pentazocine, and the third muscarine application was after a ~ 30-s exposure to σ_1 receptor antagonist haloperidol plus (+)-pentazocine. In some experiments, this order of pre-exposure to σ_1 receptor ligands was changed, that is, pre-exposure to 10 μM (+)-pentazocine preceded the third instead of the second application of 1 μM muscarine. Fig. 6A showed representative responses of five cells loaded with fura-2/AM in an experiment. Summarized data in Fig. 6B showed that 10 and 50 μM haloperidol, when co-incubated with 10 μM (+)-pentazocine, were not able to reverse the reduction of muscarine-induced $[Ca^{2+}]_i$ changes by (+)-pentazocine. In SH-SY5Y cell populations resuspended in BSS–HEPES buffer, 50 μM haloperidol also did not reverse the reduction of 5 μM muscarine-induced $[Ca^{2+}]_i$ changes by low micromolar concentrations (1–10 μM) of (+)-pentazocine or BD737, although 50 μM haloperidol alone caused a slight increase of basal $[Ca^{2+}]_i$ (Fig. 2).

3.4. The affinities of σ_1 receptor ligands for muscarinic receptors measured by radioligand binding

Because we did not observe antagonism of (+)-pentazocine and BD737 by haloperidol in the above experiments, we tested the affinities of these drugs for muscarinic receptors in radioligand binding studies. The binding assays were done using SH-SY5Y whole-cell preparations in BSS–HEPES buffer, with muscarinic receptors labeled by 0.5 nM [^{3}H]quinuclidinyl benzilate (Fig. 7). The K_d value of [^{3}H]quinuclidinyl benzilate for muscarinic receptors in SH-SY5Y cells was reported to be 25 pM (Serra et al., 1988). This value was used to compute the binding affinities of σ_1 receptor ligands for muscarinic receptors with the MacLigand 4.97 program. Computer fitted K_i values for (+)-pentazocine, BD737 and haloperidol were 0.51 ± 0.06, 0.32 ± 0.07 and 4.4 ± 2.3 μM, respectively. Non-specific binding measured in the presence of 1 μM atropine was typically 5% of total binding.



	K_i (μM)	
(+)-Pentazocine	0.51 ± 0.06	n = 3
BD737	0.32 ± 0.07	n = 3
Haloperidol	4.4 ± 2.3	n = 3

Fig. 7. Competition for [^{3}H]quinuclidinyl benzilate by σ receptor ligands in SH-SY5Y whole-cell preparations. Shown were representative competition binding curves from a single experiment with triplicates (mean ± S.D.) analyzed with the GraphPad Prism 3.0 program. K_i values from pooled experiments of the indicated numbers (n) were determined with the MacLigand 4.97 program. The K_d value of [^{3}H]quinuclidinyl benzilate for muscarinic receptors used in analysis was 25 pM. The concentration of [^{3}H]quinuclidinyl benzilate used in experiments was ~0.5 nM. Non-specific binding measured in the presence of 1 μM atropine was typically 5% of total binding.

4. Discussion

Our radioligand binding study is to our knowledge the first to show that σ_1 receptors are present in SH-SY5Y cells. K_i values for (+)-pentazocine (67 ± 10 nM) and BD737 (14 ± 1.0 nM) measured in whole-cell preparations were slightly greater that those in cell homogenates. This was likely caused by disruption and reconstitution of cellular membrane structures during cell homogenate preparations. Compared with K_i values [(+)-pentazocine, 9.5 ± 1.0 nM; BD737, 4.7 ± 0.7 nM] obtained from similar binding assays done in rodent cerebellar homogenates (Hong and Werling, 2000), the K_i values of (+)-pentazocine were considerably greater, suggesting the (+)-pentazocine-sensitive [^{3}H]haloperidol binding sites in SH-SY5Y cells were slightly different from those in rodent brain homogenates. Total binding measured in the absence and presence of 100 nM σ_2 receptor antagonist BIMU-8 was about the same level, suggesting that σ_1 sites accounted for the majority of σ-like sites in SH-SY5Y cells.

SH-SY5Y cells were reported to express mainly the M_3 subtype muscarinic receptors (Lambert et al., 1989). The presence of M_1 receptors was also shown (Kukkonen et al., 1992). It has been well characterized that the main signal transduction events following M_1 and M_3 receptor activation include phosphoinositide hydrolysis and rapid $[Ca^{2+}]_i$ increase resulting from mobilization of intracellular calcium from IP_3-sensitive endoplasmic reticulum Ca^{2+} stores (Felder, 1995). We first studied the characteristics of $[Ca^{2+}]_i$ changes in SH-SY5Y cells following muscarine application. Muscarine-induced $[Ca^{2+}]_i$ changes usually exhibited a pattern of initial fast-rising peaks followed by gradual decrease if the stimulus was sustained (Fig. 2). In some cells, sustained muscarine stimulation resulted in oscillatory $[Ca^{2+}]_i$ changes with a decreasing magnitude. The oscillatory responses were similar to that observed in cultured rat hippocampal neurons after muscarinic receptor activation (Irving and Collingridge, 1998). Prolonged agonist stimulation has been shown to cause rapid desensitization and internalization of muscarinic receptors (Wojcikiewicz et al., 1993). In the study, we chose to use a short application of muscarine to induce just the initial $[Ca^{2+}]_i$ peaks. This

muscarine-induced initial $[Ca^{2+}]_i$ peaks showed a dose–response relationship in SH-SY5Y cells (Fig. 3).

In our experimental paradigms, SH-SY5Y cells were stimulated with muscarine three or four times. Between each application of muscarine, there was a 2- to 3-min recovery period when cells were perfused with BSS–HEPES buffer. We estimated the extent of muscarinic receptor desensitization by comparing $[Ca^{2+}]_i$ changes induced by the first and last applications of muscarine in the same cells. On average, the $[Ca^{2+}]_i$ responses induced by the last application of muscarine were more than 70% of that induced by the first application (Figs. 4–6).

When applied alone, σ_1 receptor ligands (+)-pentazocine, BD737 or haloperidol up to 10 μM did not significantly alter $[Ca^{2+}]_i$ in SH-SY5Y cell suspension and single cell $[Ca^{2+}]_i$ measurement. At concentrations greater than 1 μM, these ligands could also potentially act on σ_2 receptors, although they had considerably lower affinities for σ_2 [K_i values of (+)-pentazocine: 6.61 μM, BD737: 68.3 nM, haloperidol: 221 nM, see Vilner and Bowen, 2000] than σ_1 receptor. Because our results of radioligand binding experiments suggested that σ_1 sites accounted for the majority of σ-like sites in SH-SY5Y cells, we assumed that these ligands mainly acted on σ_1 receptors. Scarcity of σ_2 receptors might also explain that BD737 or haloperidol at 10 μM did not significantly affect $[Ca^{2+}]_i$ in SH-SY5Y cells, different from the observations in SK-N-SH cells by Vilner and Bowen (2000). They reported that high concentrations (10–100 μM) of BD737 and haloperidol caused a substantial $[Ca^{2+}]_i$ rise in SK-N-SH cells, which were likely mediated by σ_2 receptors. As seen in this case, it is not unusual to see σ receptor ligands produce responses only at concentrations several fold higher than their K_d values.

We observed that muscarine-induced $[Ca^{2+}]_i$ changes were substantially reduced, following pre-exposure to (+)-pentazocine or BD737 for approximately 30 s. The inhibition by (+)-pentazocine or BD737 was concentration related (Figs. 4 and 5). No substantial reduction of muscarine-induced $[Ca^{2+}]_i$ responses was observed following pre-exposure of nanomolar concentrations of these ligands (data not shown). Since the reduced $[Ca^{2+}]_i$ responses were significantly different from those of the last muscarine application, this effect could not be completely caused by muscarinic receptor desensitization. The reduction of muscarine-induced $[Ca^{2+}]_i$ changes was unlikely due to non-specific effects of σ receptor ligands because these σ receptor ligands alone did not affect basal $[Ca^{2+}]_i$ levels at concentrations up to 10 μM.

We then examined whether the inhibitory effects on muscarine-induced $[Ca^{2+}]_i$ changes by (+)-pentazocine and BD737 could be reversed by application of the σ receptor antagonist haloperidol. When 10 or 50 μM haloperidol was co-applied with 10 μM (+)-pentazocine, reduction of muscarine-induced $[Ca^{2+}]_i$ changes was not affected (Fig. 6). Although 50 μM haloperidol alone caused a slight increase of $[Ca^{2+}]_i$ in SH-SY5Y cell populations resuspended in BSS–HEPES buffer, it also did not reverse the reduction of 5 μM muscarine-induced $[Ca^{2+}]_i$ changes by low micromolar concentrations of (+)-pentazocine (Fig. 2) or BD737 (data not shown). These results prompted us to suspect that the inhibitory effects of (+)-pentazocine or BD737 might not be directly mediated through σ_1 receptors. We subsequently found that (+)-pentazocine, BD737 and haloperidol had submicromolar K_i values for muscarinic receptors labeled with [^{3}H]quinuclidinyl benzilate in SH-SY5Y cells. Thus, micromolar concentrations of (+)-pentazocine and BD737 could compete for binding to a large fraction of muscarinic receptors in $[Ca^{2+}]_i$ measurement experiments, resulting in reduced interaction of muscarine with muscarinic receptors and decreased $[Ca^{2+}]_i$ responses.

Interactions of σ receptor ligands with muscarinic agonist-stimulated phosphoinositide turnover have been reported by Bowen et al. (1988). Micromolar concentrations of (+)-pentazocine, 1,3-di-*o*-tolylguanidine (DTG) and haloperidol were shown to inhibit carbachol-stimulated phosphoinositide turnover in rat brain synaptosome preparations, whereas no significant effects on phosphoinositide turnover by these σ receptor ligands alone were observed. After noticing a strong correlation between σ-binding affinity of σ receptor ligands and their potency at inhibiting carbachol-induced phosphoinositide turnover, they proposed that the inhibitory effects might be mediated via σ receptors (Bowen et al., 1993).

Here we studied effects of σ_1 receptor ligands on $[Ca^{2+}]_i$ changes following muscarinic receptor activation in human neuroblastoma SH-SY5Y cells. The reduction of muscarine-induced Ca^{2+} changes by (+)-pentazocine and BD737 we observed in SH-SY5Y cells was consistent with the inhibitory effects of micromolar concentrations of σ_1 receptor ligands on carbachol-stimulated phosphoinositide turnover. Since the fast-rising $[Ca^{2+}]_i$ peaks following muscarinic receptor activation were due to Ca^{2+} release from IP_3-sensitive endoplasmic reticulum Ca^{2+} stores, lower $[Ca^{2+}]_i$ responses could be resulting from reduced phosphoinositide hydrolysis. Based on our results from radioligand binding studies measuring the affinities of σ receptor ligands for muscarinic receptors, we believe the inhibitory effect of σ receptor ligands was likely not mediated through σ receptors. The K_i values of (+)pentazocine and BD737 for [^{3}H]quinuclidinyl benzilate-labeled muscarinic receptors in SH-SY5Y cells were less than 1 μM. Micromolar concentrations of these drugs would be likely to block muscarinic receptors under our experimental conditions. Others have also reported that pentazocine and haloperidol inhibited [^{3}H]quinuclidinyl benzilate or [^{3}H]pirenzepine binding with IC_{50} values between 0.5 and 10 μM in rodent brain homogenates (Candura et al., 1990; Hudkins and DeHaven-Hudkins, 1991).

In this study, we were not able to determine whether activation of σ_1 receptors had any direct effect on IP_3-induced Ca^{2+} release from endoplasmic reticulum in SH-SY5Y cells. Possible effects of (+)-pentazocine and BD737

on IP_3-mediated Ca^{2+} release following σ_1 receptor activation might be masked by the inhibition of $[Ca^{2+}]_i$ response due to muscarinic receptor blockade. Hayashi et al. (2000) reported that nanomolar concentrations of (+)-pentazocine potentiated bradykinin-induced $[Ca^{2+}]_i$ increase in NG-108 cells, and the potentiation was blocked by the σ_1 receptor antagonist NE-100. Interestingly, a minimum 10-min preincubation of (+)-pentazocine was required to induce significant potentiation in their experiments. The duration of exposure to (+)-pentazocine and BD737 in our experiments was only about half a minute, which likely did not have significant direct effects on Ca^{2+} release from IP_3-sensitive endoplasmic reticulum Ca^{2+} stores.

It is worth noting that accumulating evidence has suggested that activation of σ receptors could affect phosphoinositide turnover and Ca^{2+} release from endoplasmic reticulum. In cultured rat cardiac myocytes, nanomolar concentrations of BD737 were shown to produce a rapid increase of IP_3 production and potentiation of Ca^{2+} transients (Novakova et al., 1998). In neuroblastoma SK-N-SH cells, micromolar concentrations (10–100 μM) of BD737 and haloperidol were found to elicit a transient $[Ca^{2+}]_i$ rise that was sensitive to thapsigargin pretreatment (Vilner and Bowen, 2000). Following their study on effects of (+)-pentazocine on bradykinin-induced $[Ca^{2+}]_i$ changes, Hayashi and Su (2001) further reported that nanomolar concentrations of (+)-pentazocine caused an ankyrin B isoform to dissociate from type III IP_3 receptors, thus presumably regulating Ca^{2+} efflux from endoplasmic reticulum. It is very tempting to speculate that regulation of Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores is involved in the signal transduction of σ receptors. Such possible modulatory mechanisms of σ receptors need to be further investigated.

In conclusion, we have shown that σ_1 receptors are present on human neuroblastoma SH-SY5Y cells. Micromolar concentrations of σ_1 receptor ligands (+)-pentazocine and BD737 reduce $[Ca^{2+}]_i$ responses induced by muscarinic receptor activation. However, this inhibitory effect is likely due to blockade of muscarinic receptors by (+)-pentazocine and BD737.

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