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Possible expression of a σ_1 site in rat pheochromocytoma (PC12) cells

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Abstract

To examine the functional interaction between the σ binding sites and nicotinic acetylcholine receptors, we investigated the effects of various σ receptor ligands on nicotine-evoked Ca^{2+} uptake in differentiated PC12 cells. The IC_{50} values of σ receptor ligands tested in this uptake study did not correlate with their K_i values in the [^{3}H]1,3-di(2-tolyl)guanidine ([^{3}H]DTG) binding to guinea pig brain reported by Rothman et al. (1991). To clarify further the binding characteristics of the σ binding sites on PC12 cells, we examined the effects of σ receptor ligands on [^{3}H]*N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine HCl ([^{3}H]NE-100) binding to PC12 membranes. The K_i values of the various drugs tested for [^{3}H]NE-100 binding site closely correlated with their K_i values for the DTG site-1 reported by Rothman et al. (1991). This study showed that PC12 cells express σ_1-like sites and the inhibitory effect of σ receptor ligands on the nicotine-evoked Ca^{2+} uptake was not directly coupled with either the σ_1 or σ_2 sites.

Keywords: Pheochromocytoma (PC12) cell; Ca^{2+} uptake; Nicotine; NE-100 (*N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine HCl); Binding assay; σ Receptor

1. Introduction

Recently, based on data obtained from biochemical and radioligand binding experiments, subtypes of σ binding sites have been classified into at least two types designated σ_1 and σ_2, which were suggested for the guinea pig brain traditional σ and the rat pheochromocytoma (PC12) cell σ-like sites, respectively (Hellewell and Bowen, 1990). The most apparent difference between the sites is that the dextrorotatory isomer of benzomorphan is several-fold more potent at σ_1 sites than their corresponding levorotatory isomers, and σ_2 sites possess low or reversed stereoselectivity (Hellewell and Bowen, 1990; Su, 1993).

Although the functional roles of σ sites have not been determined, several reports are available. Paul et al. (1993) have reported that σ receptor ligands noncompetitively inhibit nicotine-stimulated catecholamine release from bovine adrenal chromaffin cells in a concentration-dependent and reversible manner and that the rank order of potency of σ receptor ligands for the inhibition of catecholamine release significantly correlates with that observed in [^{3}H](+)-pentazocine binding selective for the σ_1 receptor subtype. Radioligand binding study (Izenwasser et al., 1993) has shown that cocaine and several σ receptor ligands inhibit dopamine uptake in the rat caudate-putamen through a common site, although the identity of this site remains under investigation. Moreover, σ receptor ligands, such as (+)-pentazocine and 1,3-di(2-tolyl)guanidine (DTG), inhibit muscarinic receptor-mediated phosphoinositide metabolism in the rat brain (Candura et al., 1990). This report is supported by our finding (Yamamoto et al., 1995) that *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine HCl (NE-100), a potent and highly selective σ_1 receptor ligand (Chaki et al., 1994), inhibited carbachol-induced inositol 1,4,5-triphosphate (IP_3) formation in a dose-dependent manner through σ_1 sites. A behavioral study (Maurice et al., 1994), in which DTG significantly reversed mecamylamine-induced amnesia in mice, suggests that σ receptor

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ligands modulate nicotinic cholinergic-dependent cognitive function. However, the functional role of σ sites remains elusive.

A clonal cell line of PC12 cells shares neural crest origins with neurons of the autonomic nervous system and functionally resembles sympathetic neurons (Greene and Tischler, 1976). Upon exposure to N^6,O^2-dibutyryl cAMP (db-cAMP), PC12 cells increase cellular adhesion and neurite outgrowth (Schubert and Whitlock, 1977). Time-lapse cinematography demonstrates that both db-cAMP and nerve growth factor have unique effects on cellular morphology (Gunning et al., 1981). PC12 cells express nicotinic acetylcholine receptors resembling neuronal nicotinic acetylcholine receptors in terms of resistance to α-bungarotoxin blockade (Patrick and Stallcup, 1977) and in cross-reactivity with monoclonal antibodies to brain nicotinic acetylcholine receptors (Whiting et al., 1987). The 'neuronal-type' of the nicotinic acetylcholine receptor channel complex of PC12 cells is more permeable to Ca^{2+} than the 'muscle-type' (Sands and Barish, 1991). Nicotinic binding to nicotinic acetylcholine receptors promotes the influx of Na^+ and Ca^{2+} (Stallcup, 1979) and a K^+ efflux (Lukas and Cullen, 1988; Lukas, 1989) in PC12 cells, presumably through nonselective cation channels associated with nicotinic acetylcholine receptors.

We examined the effects of various σ receptor ligands upon Ca^{2+} uptake through nicotinic acetylcholine receptors in db-cAMP-differentiated PC12 cells, to reveal the functional interaction between the σ binding sites and nicotinic acetylcholine receptors. Furthermore, to clarify the characteristics of the σ binding sites on PC12 cells, we studied the binding of [^{3}H]NE-100 to membrane fractions derived from PC12 cells.

2. Materials and methods

2.1. Materials

Fetal calf and horse sera were purchased from Cell Culture Labs (Cleveland, OH). Phencyclidine (PCP) was donated by Shionogi Pharmaceutical (Osaka, Japan). NE-100, [^{3}H]NE-100 (specific activity, 3145 GBq/mmol), (+)-pentazocine, (−)-pentazocine, 1-(cyclopropylmethyl)-4-[2′-(4″-fluorophenyl)-2′-oxoethyl] piperidine HBr (DuP 734) and 4-[2′-(4″-cyanophenyl)-2′-oxoethyl]-1-(cyclopropylmethyl) piperidine (XJ 448) were synthesized in the Department of Organic Chemistry at the Research Center, Taisho Pharmaceutical (Saitama, Japan). ^{45}Ca (specific activity, 39.21 GBq/mmol) was purchased from NEN (Boston, MA). Other chemicals, such as 3-[3-hydroxyphenyl]-*N*-(1-propyl)-piperidine (3-PPP) and *N*-allylnormetazocine (SKF 10047), were obtained from commercial suppliers. DTG, haloperidol, (+)-butaclamol, (−)-butaclamol, (+)-pentazocine, (−)-pentazocine, and 1-[2-[bis(4-fluorophenyl) methoxy]ethyl]-4-(3-phenylpropyl)piperazine 2HCl (GBR 12909) were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was $< 0.3\%$. All other drugs were dissolved in distilled water (for the ^{45}Ca uptake) or 50 mM Tris-HCl buffer adjusted to pH 7.5 (for [^{3}H]NE-100 binding), except for NE-100, which was initially dissolved in distilled water, then diluted with the same buffer.

2.2. Cell culture

Undifferentiated PC12 cells were cultured in 55-cm^2 polystyrene tissue culture dishes (Falcon), containing 15 ml of growth medium in a humidified atmosphere of 95% air/5% CO_2 at 37°C. The growth medium was Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY), containing 5% fetal calf serum, 5% heat-inactivated (56°C, 30 min) horse serum and antibiotics (100 ng/ml streptomycin and 50 U/ml penicillin G). The medium was replaced every 3 days and the cells were subcultured weekly. For $^{45}Ca^{2+}$ uptake experiments, 96-well plastic plates, double-coated with poly-L-lysine and acid-soluble rat tail collagen, were used for subculturing. After replating at a density of $2–3 \times 10^4$ cells/cm^2, subconfluent cells were differentiated for 4 days with 200 ng/ml db-cAMP and the medium was replaced every 2nd day. Neurite formation was visible after 2 days, and by 4 days, over 70% of cells exhibited processes 4-fold longer than the diameter of one cell body.

2.3. Ca^{2+} uptake assays

The differentiated cells on the 96-well plates were incubated in 50 μl of fresh DMEM with or without drugs for 30 min under conditions described above. Thereafter, 25 μl of the same medium including ^{45}Ca (300–400 kdpm/well) or (−)-nicotine (final concentration 100 μM for stimulation) was added and the cells were further incubated for 2 min. After the uptake reaction was terminated by discarding the bathing medium, the cells were immediately washed with modified Locke's medium (composition in mmol/l: NaCl, 159.6; $CaCl_2$, 1.8; sucrose, 1; $NaHCO_3$, 6; glucose, 10; and Hepes, 2; pH 7.2) for 4 ×, and stored at −20°C. The cells were solubilized with 1% Triton X-100 and then incubated at 37°C for 60 min. The solubilized radioactivity was counted by liquid scintillation spectrometry (Beckman LS 9800) (Beckman, Fullerton, CA) in 3 ml of Aquasol II (NEN, Boston, MA), with a counting efficiency of 45–50%. The protein content was determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

2.4. Membrane preparation

Undifferentiated PC12 cells were homogenized in 20 vols. of ice-cold 50 mM Tris–HCl buffer (pH 7.5; standard buffer) using a motor-driven homogenizer of the Potter

type and centrifuged at 20000 × *g* for 20 min at 4°C. The membrane fraction was suspended in 20 vols. of the standard buffer and then centrifuged in the same manner. The pellet was quickly frozen, and stored at −80°C prior to the binding assay. On the day of the experiment, the membrane was resuspended in the standard buffer. The protein concentration in the final suspension was ~0.8 mg/ml.

2.5. [^{3}H]NE-100 binding assays

The binding reaction was initiated by adding membrane suspensions (50 μg of protein/well) to the standard buffer containing [^{3}H]NE-100 (final 0.8–1.6 nM) in a final volume of 0.2 ml. This mixture was then incubated for 90 min at 25°C. The reaction was terminated by rapid passage through Whatman GF/C filters using an Inotech 96 channel cell harvester (Wohlen, Switzerland) under reduced pressure. The filters were washed 10 × with the standard buffer cooled at 4°C. Absorption of [^{3}H]NE-100 binding to the filters was reduced by soaking them in 0.5% polyethyleneimine for at least 4 h at 25°C beforehand and nonspecific binding was defined in the presence of 5 or 10 μM haloperidol. The radioactivity and the protein concentration were determined as described above.

2.6. Data analysis and statistics

The 50% inhibitory concentrations (IC_{50}) and Hill coefficient values in the ^{45}Ca uptake assay were calculated by means of iterative curve fitting using the InPlot program (GraphPad Software, San Diego, CA). The inhibition constants (K_i) and Hill coefficient values in the [^{3}H]NE-100 binding assay were calculated using the LIGAND program (Elsevier-BIOSOFT, Cambridge, UK).

3. Results

3.1. Effects of σ receptor ligands on nicotine-evoked Ca^{2+} uptake in differentiated PC12 cells

The IC_{50} values and the Hill coefficients are summarized in Table 1. All the test drugs dose-dependently inhibited nicotine-evoked Ca^{2+} uptake in the differentiated PC12 cells. The rank order of potency that inhibited nicotine-evoked Ca^{2+} uptake was: NE-100, PCP, dextromethorphan, and GBR 12909 > haloperidol, (+)-butaclamol, and (−)-butaclamol > (+)-pentazocine, DTG, (−)-SKF 10047, and (+)-SKF10047 > DuP 734, (−)-pentazocine, XJ 448, and (−)-3-PPP ≫ (+)-3-PPP. DuP 734, (−)-pentazocine, XJ 448, (−)-3-PPP, and (+)-3-PPP did not fully inhibit $^{45}Ca^{2+}$ uptake even at a concentration of 30 μM (51.4 ± 15.8%, 65.2 ± 7.0%, 68.9 ± 10.7%, 61.4 ± 7.9%, and 76.2 ± 8.4% of the nicotine-evoked uptake level, respectively). None of the drugs tested affected the basal Ca^{2+} uptake.

Table 1
Effects of test drugs on nicotine-evoked Ca^{2+} uptake in differentiated PC12 cells

Drugs	IC_{50} (μM)	Hill coefficient
DTG	16.1 ± 8.2	1.04 ± 0.14
Haloperidol	3.1 ± 0.7	1.55 ± 0.47
(+)-3PPP	298 ± 143	0.61 ± 0.32
(−)-3PPP	78.6 ± 39.9	1.08 ± 0.23
(+)-SKF 10047	22.5 ± 9.7	0.88 ± 0.26
(−)-SKF 10047	17.6 ± 7.5	0.66 ± 0.02
(+)-Pentazocine	13.2 ± 1.9	0.78 ± 0.16
(−)-Pentazocine	60.8 ± 25.1	1.06 ± 0.52
(+)-Butaclamol	4.5 ± 0.8	1.16 ± 0.44
(−)-Butaclamol	8.1 ± 1.9	0.67 ± 0.03
Dextromethorphan	1.9 ± 0.5	1.40 ± 0.15
PCP	1.2 ± 0.4	1.03 ± 0.05
DuP 734	27.6 ± 8.9	0.72 ± 0.14
XJ 448	75.6 ± 52.0	2.06 ± 1.19
NE-100	0.41 ± 0.26	1.95 ± 0.24
GBR12909	3.2 ± 1.4	1.23 ± 0.32

Ca^{2+} uptake assays were performed in quintuplicate. Data are expressed as mean ± S.E.M. of three independent experiments.

The IC_{50} values of the various drugs in this nicotine-evoked $^{45}Ca^{2+}$ uptake study were compared with their K_i values in [^{3}H]DTG binding to guinea pig brain reported by Rothman et al. (1991). There was no correlation between the IC_{50} and K_i values for DTG site-1 or site-2.

3.2. [^{3}H]NE-100 binding to PC12 cell membranes

Incubation of various concentrations of the membrane fraction with [^{3}H]NE-100 indicated that binding of the radioligand was linear with membrane concentrations up to ~4.5 mg wet weight (~75 μg of protein)/0.2 ml assay volume. The specific [^{3}H]NE-100 binding was time-dependent, equilibrium being reached within 60 min. Thus, subsequent [^{3}H]NE-100 binding assays were performed under the optimal conditions described in Section 2. The total binding was ~2500–5000 dpm/well. Nonspecific binding represented only 5.7–9.8% of the total binding at 1.6 nM [^{3}H]NE-100. The K_d value was 14.5 ± 3.5 nM and the B_{max} value was 3.9 ± 0.8 pmol/mg of protein.

We examined the ability of various drugs to inhibit [^{3}H]NE-100 binding to the homogenates derived from undifferentiated PC12 cells. The K_i values and the slope factors (Hill coefficients) are summarized in Table 2. All the test drugs except atropine and (−)-nicotine inhibited [^{3}H]NE-100 binding at 10 μM concentrations (Fig. 1A,B). Of all the drugs tested, NE-100 (K_i = 14.0 ± 4.0 nM) was the most powerful inhibitor of [^{3}H]NE-100 binding. The order of inhibition was: NE-100 > haloperidol and DuP 734 > (+)-pentazocine, (−)-pentazocine, and DTG > (−)-butaclamol and (+)-SKF 10047 ≫ atropine and (−)-nicotine. The representative σ_1-selective ligand,

Table 2
Effects of test drugs on [^{3}H]NE-100 binding to PC12-derived homogenates

Drugs	K_i (nM)	Hill coefficient
DTG	289 ± 23	0.86 ± 0.02
Haloperidol	34.5 ± 12.7	0.66 ± 0.05
(+)-SKF 10047	1381 ± 175	0.67 ± 0.02
(+)-Pentazocine	108 ± 31	0.53 ± 0.02
(−)-Pentazocine	144 ± 38	0.62 ± 0.02
(−)-Butaclamol	785 ± 178	0.98 ± 0.04
DuP 734	35.5 ± 4.7	1.25 ± 0.02
NE-100	14.0 ± 4.0	1.04 ± 0.11
(−)-Nicotine	152 136 ± 11 075	1.12 ± 0.04
Atropine	71 406 ± 947	1.03 ± 0.06

The [^{3}H]NE-100 binding assays were performed in triplicate. Data are expressed as mean ± S.E.M. of four independent experiments.

(+)-pentazocine, was almost as effective as its (−)-isomer. Differentiation of PC12 cells induced by treatment with 200 ng/ml db-cAMP for 4 days had no dramatic effects on [^{3}H]NE-100 binding characteristics (data not shown).

Double-log plots of the K_i values of the drugs in the [^{3}H]NE-100 binding to PC12 cell membranes against their K_i values at the [^{3}H]DTG site-1 in guinea pig brain (Rothman et al., 1991) are shown in Fig. 2. There was a highly significant correlation ($r = 0.98$, $P < 0.01$; slope = 1.44) between the drug potencies at the [^{3}H]DTG site-1 and at the [^{3}H]NE-100 binding site. Moreover, when the drug potencies at the [^{3}H]NE-100 binding sites were compared with their potencies at the [^{3}H]DuP734 binding sites (Culp et al., 1992) or at the [^{3}H](+)-pentazocine binding sites (Cagnotto et al., 1994), the following correlations were obtained: $r = 0.86$, $P < 0.05$; slope = 1.43 or $r =$ 0,92, $P < 0.01$; slope = 1.00, respectively. However, the K_i values of the various drugs in [^{3}H]NE-100 binding assay did not correlate with their IC_{50} values in the $^{45}Ca^{2+}$ uptake study ($r = 0.54$).

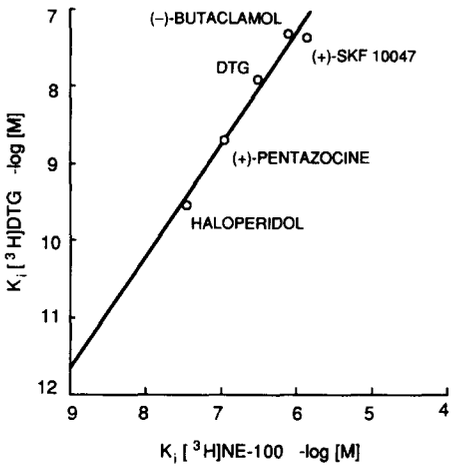

Fig. 2. Correlation of the potency of various drugs at the [^{3}H]DTG site-1 in guinea pig brain (Rothman et al., 1991) with that at the [^{3}H]NE-100 binding site in PC12 cell membranes.

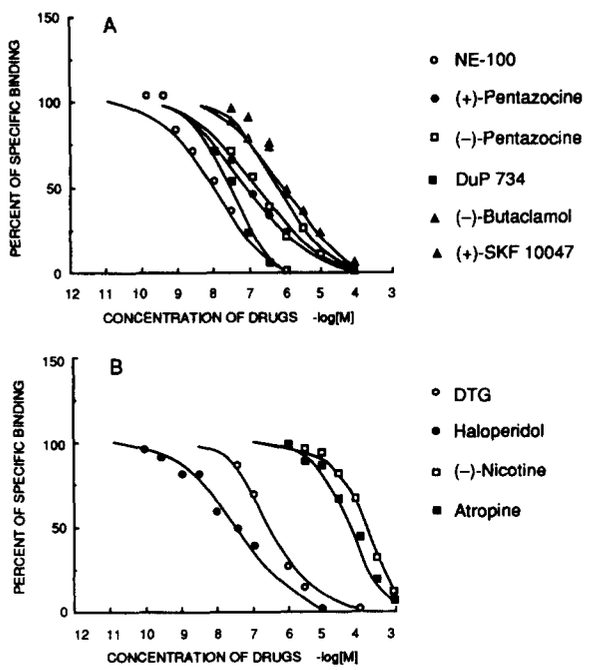

Fig. 1. Competition curves of [^{3}H]NE-100 binding to the PC12-derived homogenates in the presence of increasing concentrations of test drugs. Values are the average of four independent experiments performed in triplicate.

4. Discussion

PC12 cells possess σ_2 sites (Hellewell and Bowen, 1990). However, technical differences in culturing PC12 cells may have unpredictable influences upon their phenotypic expression. Therefore, we characterized the σ_1 sites in PC12 cells more precisely using the novel selective σ_1 receptor ligand, NE-100 (Okuyama et al., 1993). Its inhibitory effect on σ_1 sites is 55 × more potent than that on σ_2 sites (Chaki et al., 1994). We found that [^{3}H]NE-100 binding to PC12 membranes was time-dependent, linear with membrane protein concentration and highly specific, suggesting the presence of σ_1 as well as σ_2 sites in PC12 cells. The affinity of [^{3}H]NE-100 binding to PC12 cell membranes was less potent than that reported by Tanaka et al. (1995) using guinea pig brain membranes. Although the pentazocines had weak stereoselectivity for [^{3}H]NE-100 binding sites, the potency of other σ receptor ligands at this site correlated well with the affinity of typical σ receptor ligands found using in vitro [^{3}H]DTG (Rothman et al., 1991), [^{3}H]DuP 734 (Culp et al., 1992) and [^{3}H](+)-pentazocine (Cagnotto et al., 1994) binding. These pharmacological profiles of [^{3}H]NE-100 binding show that this ligand labels the σ_1-like binding site in PC12 membranes. Our studies indicated that PC12 cells possess σ_1-

like binding sites. This is of interest, since PC12 cells are a tumor cell line derived from rat adrenal chromaffin cells (Greene and Tischler, 1976), which may have originally expressed σ_1 (Paul et al., 1993) as well as σ_2 sites.

We found that there was no functional interaction between nicotinic acetylcholine receptor-mediated ^{45}Ca uptake and σ receptors in the differentiated PC12 cells and that the selective σ_1 receptor ligand NE-100 had no affinity for binding sites for agonists or antagonists in the nicotinic acetylcholine receptor. These results indicate that the σ receptor ligand affected nicotinic acetylcholine receptor-mediated ^{45}Ca uptake by means of a process that did not include σ receptors. However, it is noteworthy that the selective σ_1 receptor ligand NE-100 potently inhibited nicotinic acetylcholine receptor-mediated ^{45}Ca uptake. This finding raises the possibility that σ_1 sites may play a role in nicotinic acetylcholine receptor-mediated ^{45}Ca uptake. Therefore, further studies are necessary to identify the factors involved in nicotinic acetylcholine receptor-mediated ^{45}Ca uptake. By contrast, Paul et al. (1993) reported that in rat adrenal chromaffin cells, the rank order of potency of ligands to inhibit nicotine-stimulated catecholamine release is significantly correlated with affinity for the σ_1 receptor. They have also reported that (+)-pentazocine inhibits nicotine-stimulated increases in intracellular Ca^{2+} concentrations (Paul et al., 1993). The latter is consistent with our results that (+)-pentazocine inhibited nicotine-stimulated ^{45}Ca uptake in PC12 cells. Since we showed a similar effect of (+)-pentazocine in nicotine-stimulated Ca^{2+} movement using PC12 cells, there might be a similar relationship between nicotinic acetylcholine receptors and σ receptors in the rat adrenal chromaffin and PC12 cells. Taken together with our findings that there was no correlation between the effects of σ receptor ligands on the nicotinic acetylcholine receptor-stimulated ^{45}Ca uptake and affinity for σ_1 or σ_2 sites and that a report described the functional coupling between nicotine-stimulated catecholamine release and σ_1 sites (Paul et al., 1993), it is most likely that σ_1 sites are involved in the mechanisms of neurotransmitter release. That is, although catecholamine release is triggered by a Ca^{2+} spike during action potential, exocytosis probably exhibits a nonlinear dependence on the Ca^{2+} concentration with an apparent cooperativity of 4 (Dodge and Rahamimoff, 1967). Furthermore, Ca^{2+}-triggered exocytosis is likely to be a complex reaction involving several proteins. Thus, σ receptor ligands may express their effects through σ_1 sites by acting on some steps or proteins in the exocytosis/fusion pathway. Brent et al. (1995) have reported that σ receptor ligands inhibit the depolarization-dependent increase in phosphorylation of synapsin Ib in rat brain synaptosomes. Considering that injection of non-phosphorylated synapsin I into squid neurons inhibits neurotransmitter release (Llinas et al., 1991), it is likely that σ receptor ligands inhibit nicotinic acetylcholine receptor-stimulated catecholamine release via the dephosphorylation of synapsin I. These are highly speculative and more studies are required to clarify the physiological functions of σ sites.

In conclusion, this study showed that PC12 cells have σ_1-like sites and that the inhibitory effect of σ receptor ligands on nicotinic acetylcholine receptor-stimulated ^{45}Ca uptake was not directly coupled with either the σ_1 or σ_2 sites.

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