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Modulation of (+)-[^{3}H]Pentazocine Binding to Guinea Pig Cerebellum by Divalent Cations

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SUMMARY

The ability of cations to modulate the binding of the σ_1 receptor-selective ligand (+)-[^{3}H]pentazocine to guinea pig cerebellum was investigated. Di- and trivalent cations biphasically inhibited (+)-[^{3}H]pentazocine binding, revealing multiple affinity states. The rank order of potency of these cations (based on the high affinity component of inhibition) was $Zn^{2+} > Co^{2+} \gg La^{3+} = Ni^{2+} = Cd^{2+} = Mn^{2+} = Gd^{2+} > Ba^{2+} = Sr^{2+} \gg Mg^{2+} > Ca^{2+}$. The inhibition of 1,3-[^{3}H]di(2-tolyl)guanidine binding to the σ_2 receptor by these cations differed qualitatively and quantitatively from their effects on (+)-[^{3}H]pentazocine binding. Although monovalent cations decreased the K_d for (+)-[^{3}H]pentazocine binding, divalent cations split (+)-[^{3}H]pentazocine binding into low and high affinity components. The B_{max} of the high affinity component decreased with increasing divalent cation concentrations. Both mono- and divalent cations significantly reduced the rate of association of (+)-[^{3}H]pentazocine with the σ_1 receptor without altering the dissociation rate. (+)-[^{3}H]Pentazocine binding was not altered by guanine nucleotides or by treatment with cholera or pertussis toxins. However, nonselective cation channel blockers (cinnarizine, hydroxyzine, prenylamine, amiodarone, and proadifen) potently inhibited (+)-[^{3}H]pentazocine binding. These results indicate that physiologically relevant concentrations of divalent cations allosterically modulate (+)-[^{3}H]pentazocine binding to the σ_1 receptor, to reveal multiple affinity states. These sites do not represent σ_1 to σ_2 subtype interconversion or ternary complex formation with guanine nucleotide-binding proteins. However, the rank order of cation potency and the inhibition of binding by cation channel blockers is consistent with a potential role for σ receptors as constituents of cation channels.

High affinity, stereoselective binding sites for benzomorphan opiates exist in the central nervous system and peripheral tissues (1–5). This σ receptor is pharmacologically distinct from receptors for opiates (1, 2, 6), dopamine (3), and the NMDA receptor/ionophore complex (7–9). Recently, ligands have been developed with high affinity, stereospecificity, and selectivity for σ receptors. One such ligand is (+)-[^{3}H]pentazocine (10), which binds with high affinity and selectivity to the σ_1 receptor subtype (11, 12). Despite the development of ligands selective for σ receptor subtypes, the physiological function of these receptors remains unclear. The σ receptor has been variously described as modulating phosphatidylinositol turnover (13, 14), altering neuronal electrical activity (15–17), inhibiting ion transport in the gut (18), and suppressing nicotine-stimulated catecholamine release from chromaffin cells (19). Part of the uncertainty over the multiplicity of σ receptor actions may be attributed to the large number of structurally diverse compounds (e.g., piperazines, guanidines, butyrophenones, and phenylpiperidines) (20, 21) that display moderate to high affinities but poor specificity for the σ receptor. Thus, multiple actions of some σ receptor ligands can be observed upon administration to intact animals or in tissue preparations *in vitro* (22–24).

Studies of the regulation by cations of ligand binding to σ receptors in less well organized preparations (e.g., cells in culture or cell membranes) provide insights into the possible function of these receptors (25). Guanine nucleotides also have been reported to reduce the affinity of some ligands for the σ_1 receptor, suggesting that σ_1 receptor actions may be mediated through G protein-regulated effectors (26–28). However, the mechanism of cation effects on ligand binding to σ receptor subtypes and the potential interaction of cations with G proteins have not been studied in depth. Such information may indicate whether σ receptor subtypes are independently coupled to either G proteins, ion channels, or both.

We have investigated the ability of cations to modulate the

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; DTG, 1,3-di(2-tolyl)guanidine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Gpp(NH)p, 5-guanylylimidodiphosphate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); MDL 12330A, [*N*-(*cis*)-2-phenylcyclopentyl]azacyclotridecan-2-imine HCl; 3-PPP, 3-(2-hydroxyphenyl)-*N*-(1-propyl)piperidine; SKF 10047, *N*-allylnormetazocine; SKF 96365, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole HCl; TMB-8, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate HCl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; G protein, guanine nucleotide-binding protein; ANOVA, analysis of variance; CTX, cholera toxin; PTX, pertussis toxin.

binding of (+)-[^{3}H]pentazocine to the σ_1 receptor (29) in the guinea pig cerebellum. This region was chosen for its low density of potentially interfering receptors (such as dopaminergic, muscarinic, and NMDA receptors) (30–33), as well as for its high density of σ receptors (21, 34). Divalent cations biphasically inhibited (+)-[^{3}H]pentazocine binding to σ_1 receptors at physiologically relevant concentrations. Furthermore, (+)-[^{3}H]pentazocine binding was not significantly modulated by guanine nucleotides or G protein-modifying treatments. These observations contrast markedly with previously described interactions of ligands with the σ_1 receptor (29) and may be consistent with an involvement of σ_1 receptors in the regulation of ion channels.

Experimental Procedures

Materials. (+)-[^{3}H]Pentazocine (specific activity, 51.7 Ci/mmol) was synthesized from optically pure (+)-[^{3}H]normetazocine as previously described (10). [^{3}H]DTG (specific activity, 39.4 Ci/mmol) was obtained from DuPont/New England Nuclear (Boston, MA). Guinea pig brains were obtained from Pel-Freeze (Rodgers, AK). CTX, PTX, Tris-GTP, and $Li_4GTP\gamma S$ were obtained from Sigma (St. Louis, MO). Protein concentrations in the homogenates of guinea pig cerebellum were determined using a bicinchoninic acid protein assay kit (Pierce Chemicals, Rockford, IL).

Binding assays. (+)-[^{3}H]Pentazocine binding was assayed using previously described techniques (12). Guinea pig brains were thawed, and the cerebella were isolated, homogenized in 50 volumes of 290 mM sucrose buffered with 5 mM HEPES free acid/4.5 mM Tris free base (pH 7.8) using a Polytron (setting 6.5, 30 sec), and then centrifuged at 20,000 × *g* for 20 min at 0–4°. The pellet was resuspended in fresh buffer, washed, and then suspended in 10 volumes of 5 mM HEPES/4.5 mM Tris buffer (pH 7.8) without sucrose. Aliquots (50 μl, containing 0.09–0.10 mg of protein) were added to each assay tube along with 50 μl of (+)-[^{3}H]pentazocine (0.5–75 nM final concentration), 50 μl of haloperidol (final concentration, 10 μM), 50 μl of the appropriate salt solution, and sufficient buffer to yield a final volume of 500 μl. The assays were performed in duplicate. Incubations were carried out for 120 min at 25° and were terminated by rapid filtration, using a Brandel M-24R filtering manifold (Brandel Biomedical, Gaithersburg, MD), over Whatman GF/C filter strips that had been pretreated with 0.03% polyethylenimine. Samples were washed with two 5-ml aliquots of ice-cold 5 mM HEPES/4.5 mM Tris buffer. The radioactivity retained by the filters was measured in a Beckman LS 5801 scintillation counter, using 4 ml of Cytoscint (ICN Biochemicals, Irvine, CA).

Similar protocols were used to assess [^{3}H]DTG binding to σ_2 receptors and [^{3}H]3-PPP binding to σ receptors in the guinea pig cerebellum. A saturating concentration (10 μM) of the σ_1-selective ligand dextrallorphan was added to each assay tube containing [^{3}H]DTG (10 nM for competition assays, 1–200 nM for saturation assays), along with the appropriate aliquots of tissue, buffer, and inorganic salts. [^{3}H]3-PPP binding was determined using 20 nM [^{3}H]3-PPP, with nonspecific binding determined in the presence of 100 μM (+)-pentazocine. The [^{3}H]DTG binding assay was incubated at room temperature for 2 hr, whereas the [^{3}H]3-PPP assay was incubated at room temperature for 1 hr. Both assays were terminated according to the procedure described above.

All of the inorganic salts were diluted from 1–4 M stock solutions with the pH adjusted to 6–8 using either HEPES free acid or Tris free base. Tonicity effects on (+)-[^{3}H]pentazocine binding were determined using sucrose, with the IC_{50} for sucrose being 1.9 ± 0.1 M. Cation competition assays were performed using 5 nM (+)-[^{3}H]pentazocine in the presence of varying (10 μM to 3.2 M) ion concentrations. Saturation analysis was performed using 0.5–75 nM final concentrations of (+)-[^{3}H]pentazocine. Association kinetics were determined by measuring the specific binding of 1, 2, or 5 nM concentrations of (+)-[^{3}H]pentazocine in the presence of cations at 8 to 11 time points between 5 and 120 min. Dissociation kinetics were analyzed by incubating (+)-[^{3}H]pentazocine without exogenous ions for 120 min. Dissociation was initiated by the addition of (+)-pentazocine (final concentration, 10 μM) along with any cations at 9 to 13 time points over a 5–300-min period.

The effects of CTX and PTX on (+)-[^{3}H] pentazocine binding were determined by resuspending a pellet of homogenized guinea pig cerebellum (washed once in the HEPES/Tris-buffered sucrose) in 10 volumes of medium containing 25 mM Tris, 2 mM EDTA, 2 mM EGTA, 1 mM ADP, 10 mM thymidine, 0.1 mM ATP, 10 mM dithiothreitol, 50 μM leupeptin, 10 μg/ml aprotinin, and 2.5 μM NAD, to which were added 20 μg/ml concentrations of either CTX or PTX. The pH was adjusted to 8 with HEPES free acid and the mixture was incubated at 37° for 90 min. After incubation, 50-μl aliquots were added to a standard (+)-[^{3}H]pentazocine saturation binding assay or to single concentrations (20 nM) of [^{3}H]3-PPP.

Data analysis. Data from competition, saturation, association, and dissociation experiments were analyzed using nonlinear regression analysis (GraphPad Software, San Diego, CA) by modeling to one- and two-component models. Two-component models were accepted if their fit to the data was significantly better than that of a single-site model ($p < 0.05$), as determined using the *F* test and the runs test. Subsequent statistical comparisons of the equilibrium and nonequilibrium binding parameters were performed using ANOVA followed by Scheffe's test or Fisher's exact test or using paired *t* test where appropriate (Statview; Brain Power Inc., Calabasas, CA).

Results

Organic and inorganic anions (as their sodium salts) did not consistently alter (+)-[^{3}H]pentazocine binding to σ_1 receptors (Table 1). Anions typically inhibited (+)- [^{3}H]pentazocine binding to a maximum of 20–50%, with IC_{50} values of 50–130 mM. Those anions with I_{max} values greater than 50% (e.g., I^-, NO_2^-, and $C_2H_3O_2^-$) inhibited (+)-[^{3}H]pentazocine binding with low potencies (150–420 mM). The Hill coefficients for all the anions tested were not significantly different from 1. Because Cl^- had minimal effects on the binding of (+)-[^{3}H] pentazocine, subsequent studies of cation effects were performed using the Cl^- salts.

Contrasting with the actions of anions on (+)-[^{3}H]pentazocine binding, cations had profound effects (Fig. 1; Table 2). Monovalent cations incompletely blocked (+)-[^{3}H]pentazocine

TABLE 1

Concentration-response relationship for the modulation of (+)-[^{3}H] pentazocine binding by selected anions

(+)-[^{3}H]Pentazocine (5 nM) was incubated with 10 μM to 3.2 M concentrations of organic and inorganic anion salts of sodium. I_{min}, minimum inhibition of (+)-[^{3}H] pentazocine binding; I_{max}, maximum inhibition of (+)-[^{3}H]pentazocine binding; IC_{50}, ion concentration inhibiting 50% of (+)-[^{3}H]pentazocine binding; n_H, Hill coefficient. I_{min}, I_{max}, IC_{50}, and n_H were determined by nonlinear regression analysis of competition data by fitting to a sigmoidal curve model. None of the variables was constrained in the fit. Each value represents the mean of three to six observations.

Anion	I_{min}	I_{max}	IC_{50}	n_H
	% of inhibition	% of inhibition	mM	
F^-	0.8 ± 2.4	23 ± 1.3	89 ± 17	1.3 ± 0.2
Cl^-	−4.0 ± 0.8	31 ± 0.8	63 ± 1.2	1.6 ± 0.4
I^-	2.9 ± 3.9	110 ± 2.7[a]	420 ± 42[a]	0.8 ± 0.1
Br^-	−2.6 ± 2.4	39 ± 2.3	130 ± 6.4	1.0 ± 0.1
NO_2^-	0.1 ± 1.3	70 ± 3.8[a]	200 ± 8.5	0.8 ± 0.1
SO_4^{2-}	−0.8 ± 8.0	35 ± 4.0	51 ± 16	1.4 ± 0.2
PO_4^{2-}	6.0 ± 1.9	67 ± 1.6[a]	46 ± 11	0.9 ± 0.1
$C_6H_5O_7^-$	−2.8 ± 3.0	53 ± 9.0	150 ± 39	1.1 ± 0.3
$C_2H_3O_2^-$	5.0 ± 2.5	100 ± 1.3[a]	150 ± 4.3	1.1 ± 0.1

[a] Significantly different from chloride, $p < 0.05$, by Scheffe's test after ANOVA.

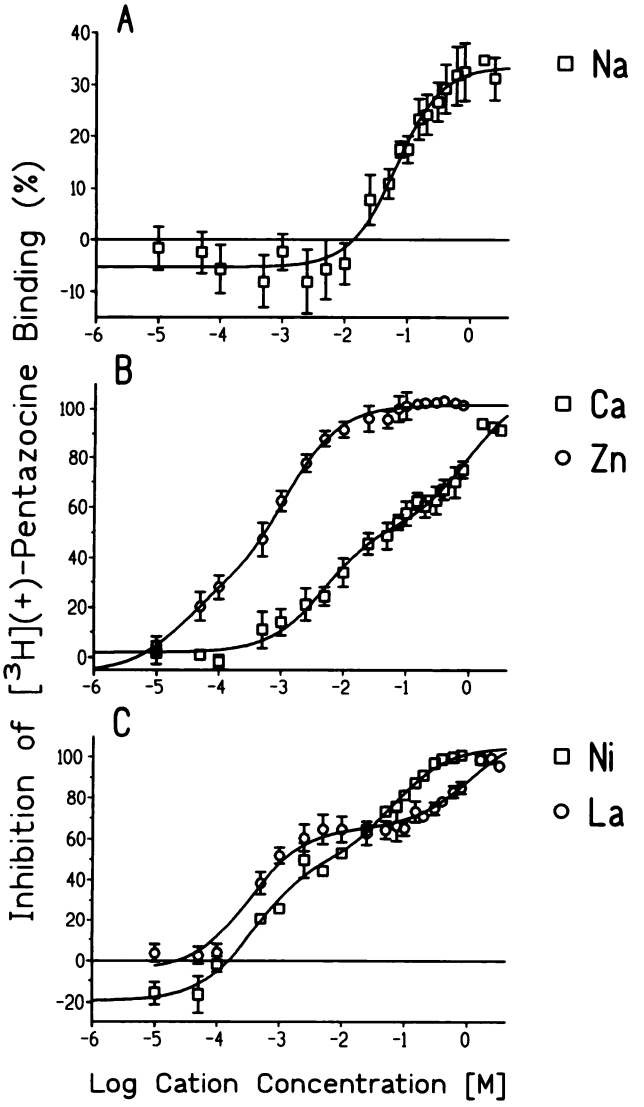

Fig. 1. Competition curves for the inhibition of (+)-[^{3}H]pentazocine binding to σ_1 receptors in guinea pig cerebellum. A, (+)-[^{3}H]Pentazocine binding is modestly enhanced at low Na^+ concentrations and incompletely inhibited at the highest Na^+ concentrations. B, Ca^{2+} and Zn^{2+} completely inhibited (+)-[^{3}H]pentazocine binding, revealing two binding components. C, Effects of Ni^{2+} and La^{3+} on (+)-[^{3}H]pentazocine binding. Note the strong accentuation of (+)-[^{3}H]pentazocine binding by low concentrations of Ni^{2+} and the pronounced differences in affinities of the two (+)-[^{3}H]pentazocine binding components in the presence of La^{3+}.

binding. The maximum inhibition observed with the Cl^- salts of Li^+, Na^+, K^+, and Cs^+ was approximately 30%, with IC_{50} values ranging from 20 to 60 mM. The Hill coefficients for monovalent cation competition with (+)-[^{3}H]pentazocine were not significantly different from 1. However, all of the di- and trivalent cations tested completely inhibited (+)-[^{3}H]pentazocine binding. A moderate enhancement of binding was observed at low concentrations (10–100 μM) of Ni^{2+} and Co^{2+}. In addition, the Hill coefficients in these competition assays were consistently and significantly less than 1 ($p < 0.05$). Subsequent analysis showed that the data fit a two-site model significantly better ($p < 0.05$) than a one-site model (Fig. 1; Table 2). The rank order of potency for the di- and trivalent cations as inhibitors of (+)-[^{3}H]pentazocine binding was $Zn^{2+} > Co^{2+} \gg La^{3+} = Ni^{2+} = Cd^{2+} = Mn^{2+} = Gd^{2+} > Ba^{2+} = Sr^{2+} \gg Mg^{2+} > Ca^{2+}$. The inhibition of [^{3}H]DTG binding to the σ_2 receptor by cations differed qualitatively and quantitatively from their effects on (+)-[^{3}H]pentazocine binding (Table 3). The monovalent cations (Li^+, Na^+, K^+, and Cs^+) were fully efficacious in inhibiting [^{3}H]DTG binding. Divalent cations potently blocked [^{3}H]DTG binding, but not all divalent cations (Sr^{2+}, Ba^{2+}, Cd^{2+}, Zn^{2+}, and Co^{2+}) inhibited binding in a biphasic fashion. The rank order of potency for the di- and trivalent cations as inhibitors of (+)-[^{3}H]pentazocine binding was $Ca^{2+} > Zn^{2+} > La^{3+} = Ni^{2+} = Mn^{2+} = Co^{2+} > Ba^{2+} = Gd^{2+} > Sr^{2+} > Mg^{2+} = Cd^{2+}$.

Saturation binding assays indicated that (+)-[^{3}H]pentazocine bound with a K_d of 3.7 nM and a B_{max} of 2.4 pmol/mg of protein (Fig. 2; Table 4) under control conditions. Na^+ monophasically decreased the affinity of (+)-[^{3}H]pentazocine for the σ_1 receptor without altering B_{max}. Divalent cations such as Mg^{2+} or Ca^{2+} also increased the K_d for (+)-[^{3}H]pentazocine binding when the data were analyzed according to a single-site model. However, when analyzed according to a two-site model, physiologically relevant concentrations of divalent cations and Krebs-Ringer medium were found to split (+)-[^{3}H]pentazocine binding into high (≈2 nM) and low (≈20–60 nM) affinity components. The B_{max} of the high affinity component decreased with increasing divalent cation concentrations. All high affinity (+)-[^{3}H]pentazocine binding was lost at the highest concentrations of Ca^{2+} and Mg^{2+} tested (660 and 1400 mM, respectively). Similarly, saturation analysis of [^{3}H]DTG binding demonstrated that selected cations decreased the B_{max} (control, 2162 ± 240 fmol/mg of protein; 10 mM Ca^{2+}, 976 ± 61 fmol/mg of protein; 300 μM Zn^{2+}, 565 ± 162 fmol/mg of protein; Krebs-Ringer, 598 ± 60 fmol/mg of protein; three experiments), without significantly altering K_d (control, 27.2 ± 6.3 nM; 10 mM Ca^{2+}, 22.8 ± 3.3 nM; 300 μM Zn^{2+}, 50.9 ± 22.5 nM; Krebs-Ringer, 20.8 ± 6.2 nM).

Mono- and divalent cations reduced the rate of (+)-[^{3}H]pentazocine association with the σ_1 receptor by 30–90%, in a concentration dependent manner (Fig. 3; Table 5). A trend toward biphasic association rates was observed in some cases (4 and 30 mM Ca^{2+} and 50 μM Zn^{2+}) (Fig. 3). None of the cations tested changed the (+)-[^{3}H]pentazocine dissociation rate (Fig. 4; Table 5).

Guanine nucleotides, alone or in combination with 2 mM Mg^{2+}, did not alter (+)-[^{3}H]pentazocine binding (Table 6). Furthermore, the inhibition of (+)-[^{3}H]pentazocine binding by Na^+, Mg^{2+}, Ca^{2+}, or Zn^{2+} was not altered by the addition of 1 mM GTP. Treatment with CTX or PTX did not significantly affect either the B_{max} (control, 2.42 ± 0.11 pmol/mg of protein, four experiments; CTX, 2.56 ± 0.17 pmol/mg of protein, three experiments; PTX, 2.32 ± 0.09 pmol/mg of protein, three experiments) or the K_d (control, 4.83 ± 1.04 nM; CTX, 5.49 ± 1.7 nM; PTX, 5.81 ± 0.84 nM) for (+)-[^{3}H]pentazocine binding under conditions that inhibited [^{3}H]3-PPP (20 nM) binding by >90% (control, 1.1 ± 0.11 pmol/mg of protein, six experiments; CTX, 28 ± 9.1 fmol/mg of protein, six experiments; PTX, 52 ± 17 fmol/mg of protein, six experiments). However, nonselective Ca^{2+} channel blockers (proadifen, prenylamine, amiodarone, hydroxyzine, and tetracaine) and inhibitors of intracellular Ca^{2+} mobilization (TMB-8 and cinnarizine) potently inhibited (+)-[^{3}H]pentazocine binding (IC_{50} = 12–190 nM) (Table 7).

TABLE 2

Concentration-response relationship for the modulation of (+)-[^{3}H]pentazocine binding by selected cations

(+)-[^{3}H]Pentazocine (5 nM) was incubated with 10 μM to 3.2 M concentrations of inorganic cation chloride salts. I_{min}, minimum inhibition of (+)-[^{3}H]pentazocine binding; I_{max}, maximum inhibition of (+)-[^{3}H]pentazocine binding; IC_{50}, ion concentration inhibiting 50% of (+)-[^{3}H]pentazocine binding (for sites A and B in the two-component model); n_H, Hill coefficient; B_A/B_B, ratio of the apparent density of sites A and B. I_{min}, I_{max}, IC_{50A+B}, and n_H were determined by nonlinear regression analysis of competition data by fitting to a sigmoidal curve model. In those cases where the n_H was less than 1, the competition data was fitted to a two-component model as well as a one-component model. When the two-component model fit the data significantly ($p < 0.05$) better than a one-component model, the IC_{50A}, IC_{50B}, and B_A/B_B values are presented. None of the variables was constrained in the fit. Each value represents the mean of four to six observations.

Cation	One-site				Two-site		
	I_{min}	I_{max}	IC_{50}	n_H	IC_{50A}	IC_{50B}	B_A/B_B
	% of inhibition	% of inhibition	mM		mM	mM	
Li^+	0.5 ± 0.9	39 ± 1.2	19 ± 1.1	0.95 ± 0.09			
Na^+	−4.0 ± 0.8	31 ± 3.3	63 ± 1.2	1.6 ± 0.41			
K^+	−7.3 ± 0.8	30 ± 1.3	45 ± 1.1	1.4 ± 0.19			
Cs^+	−14 ± 1.5	35 ± 2.0	21 ± 1.2	0.83 ± 0.11			
Mg^{2+}	0.1 ± 0.5	100 ± 2.9		0.36 ± 0.03	1.8 ± 0.93	650 ± 22	46/54
Ca^{2+}	1.9 ± 1.8	110 ± 7.4		0.44 ± 0.02	2.4 ± 0.39	490 ± 29	53/47
Sr^{2+}	0.3 ± 3.1	100 ± 4.4		0.37 ± 0.03	0.69 ± 0.66	410 ± 21	48/52
Ba^{2+}	−3.1 ± 2.6	110 ± 4.3		0.47 ± 0.04	0.58 ± 0.41	160 ± 8.6	39/61
Mn^{2+}	0.1 ± 2.1	120 ± 6.4		0.32 ± 0.03	0.48 ± 0.16	430 ± 15	55/45
Cd^{2+}	−2.5 ± 4.8	96 ± 2.0		0.78 ± 0.10	0.43 ± 0.05	99 ± 8.9	82/18
Gd^{2+}	−2.1 ± 0.6	100 ± 1.4		0.41 ± 0.03	0.51 ± 0.02	230 ± 27.3	70/30
Zn^{2+}	0.3 ± 3.5	100 ± 1.5		0.67 ± 0.05	0.05 ± 0.01	1.4 ± 0.8	34/66
Ni^{2+}	−47 ± 20	130 ± 15		0.31 ± 0.08	0.39 ± 0.11	67 ± 4.1	55/45
Co^{2+}	−37 ± 11	100 ± 3.0		0.37 ± 0.04	0.10 ± 0.01	41 ± 2.5	50/50
La^{3+}	−4.5 ± 3.4	110 ± 7.4		0.34 ± 0.04	0.33 ± 0.07	1000 ± 64	60/40

TABLE 3

Concentration-response relationship for the modulation of [^{3}H]DTG binding by selected cations

[^{3}H]DTG (10 nM) binding to σ_2 receptors in guinea pig cerebellum was determined in the presence of 10 μM dextrallorphan. Cations were added to the assay in 0.1 μM to 1 M concentrations. I_{min}, minimum inhibition of [^{3}H]DTG binding; I_{max}, maximum inhibition of [^{3}H]DTG binding; IC_{50}, ion concentration inhibiting 50% of [^{3}H]DTG binding (for sites A and B in the two-component model); n_H, Hill coefficient; B_A/B_B, ratio of the apparent density of sites A and B. I_{min}, I_{max}, IC_{50A+B}, and n_H were determined by nonlinear regression analysis of competition data by fitting to a sigmoidal curve model. In those cases where the n_H was less than 1, the competition data was fitted to a two-component model as well as a one-component model. When the two-component model fit the data significantly ($p < 0.05$) better than a one-component model, the IC_{50A}, IC_{50B}, and B_A/B_B values are presented. None of the variables was constrained in the fit. Each value represents the mean of four or five observations.

Cation	One-site				Two-site		
	I_{min}	I_{max}	IC_{50}	n_H	IC_{50A}	IC_{50B}	B_A/B_B
	% of inhibition	% of inhibition	mM		mM	mM	
Li^+	16 ± 4.4	100 ± 3.2	16 ± 5.7	1.2 ± 0.18			
Na^+	11 ± 3.7	96 ± 5.2	19 ± 2.9	0.91 ± 0.22			
K^+	−17 ± 6.8	81 ± 5.0	14 ± 1.8	0.95 ± 0.14			
Cs^+	−6.8 ± 6.9	110 ± 10	28 ± 4.7	0.90 ± 0.10			
Mg^{2+}	17 ± 3.5	90 ± 7.3		0.43 ± 0.03	0.57 ± 0.15	120 ± 43	78/24
Ca^{2+}	17 ± 4.2	93 ± 6.4		0.52 ± 0.09	0.016 ± 0.008	3.0 ± 1.3	47/53
Sr^{2+}	−6.1 ± 9.4	82 ± 10	0.44 ± 0.19	0.78 ± 0.12			
Ba^{2+}	−0.1 ± 4.9	91 ± 5.7	0.28 ± 0.07	0.98 ± 0.10			
Mn^{2+}	−12 ± 4.3	140 ± 4.4		0.52 ± 0.08	0.17 ± 0.61	22 ± 4.3	69/31
Cd^{2+}	13 ± 12	73 ± 6.2	0.63 ± 0.04	0.83 ± 0.10			
Gd^{2+}	5.4 ± 13	86 ± 9.4		0.54 ± 0.12	0.26 ± 0.05	140 ± 34	47/53
Zn^{2+}	−8.4 ± 8.9	110 ± 4.2	0.06 ± 0.01	1.53 ± 0.53			
Ni^{2+}	−6.7 ± 13	150 ± 7.7		0.59 ± 0.15	0.14 ± 0.01	300 ± 68	67/33
Co^{2+}	16 ± 12	110 ± 5.7	0.19 ± 0.04	1.10 ± 0.41			
La^{3+}	−11 ± 3.0	130 ± 2.7		0.28 ± 0.01	0.12 ± 0.05	140 ± 16	48/52

Discussion

Divalent but not monovalent cations biphasically inhibited (+)-[^{3}H]pentazocine and [^{3}H]DTG binding to σ_1 and σ_2 receptors, respectively, in the guinea pig cerebellum. Competition assays using (+)-[^{3}H]pentazocine revealed the presence of high and low affinity binding sites for divalent but not monovalent cations. In contrast, monovalent cations were more efficacious and divalent cations were more potent in inhibiting [^{3}H]DTG binding to σ_2 sites. Furthermore, not all divalent cations biphasically displaced [^{3}H]DTG from σ_2 receptors. This may reflect functional differences in cation selectivity or in cation accessibility to sites on the σ_2 receptor. The high potencies of physiologically relevant cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) in blocking [^{3}H]DTG and (+)-[^{3}H]pentazocine binding suggest that minimal ligand binding to σ receptors would occur in the normal extracellular environment. This is supported by the significant decrease in ligand affinity for σ receptors observed in physiological preparations (16, 17, 19) and in intact animals (35). However, the highest densities of σ_1 and σ_2 receptor subtypes are found in intracellular structures such as the endoplasmic reticulum (36, 37), where cation concentrations may

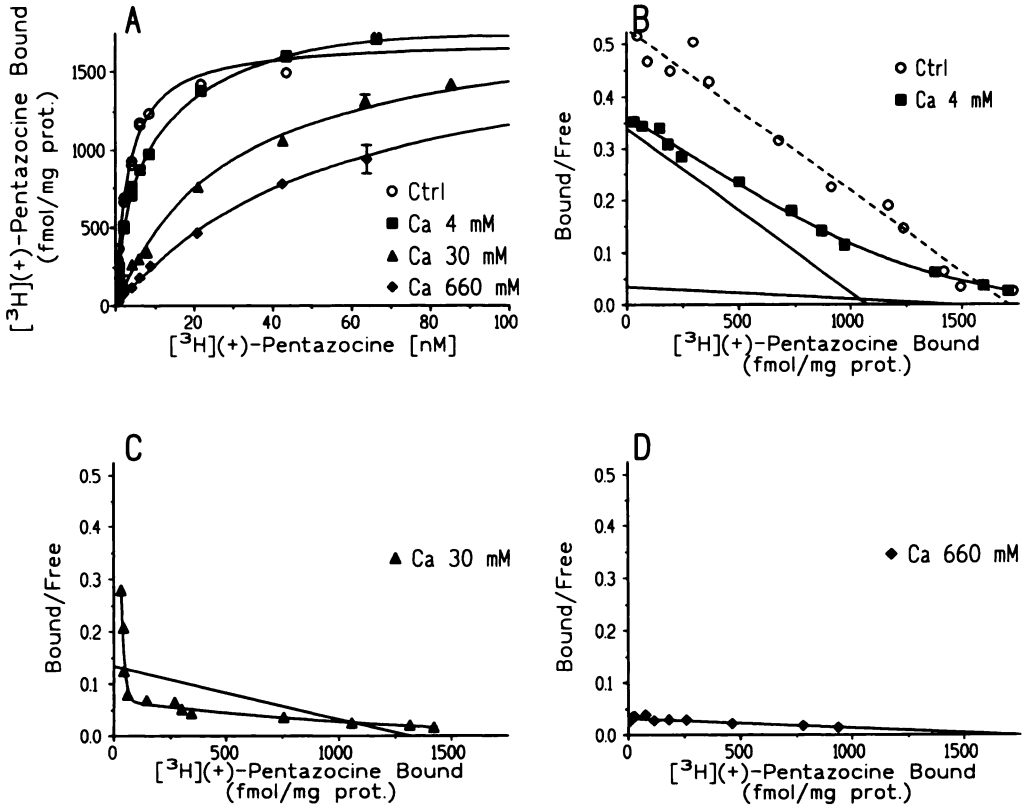

Fig. 2. Saturation binding studies of the effects of selected Ca^{2+} concentrations on the binding of (+)-[^{3}H]pentazocine to σ_1 sites in the guinea pig cerebellum. A, Binding isotherms show the effects of 4, 30, and 660 mM concentrations of Ca^{2+} on (+)-[^{3}H]pentazocine binding. Each *curve* was determined by nonlinear regression analysis using a single-component model [control (*Ctrl*), 30 and 660 mM Ca^{2+}], or a two-component model (4 mM Ca^{2+}; $F_{(10,7)} = 20.7$, $p < 0.001$). B, Scatchard plot of the control and 4 mM Ca^{2+} curves in A. The control *line* was determined by linear regression. The *curve* for the 4 mM Ca^{2+} assay was determined by polynomial regression. Nonlinear regression analysis determined the *lines* associated with the 4 mM Ca^{2+} assay. C, Scatchard plot of the 30 mM Ca^{2+} curve in A. The *curve* was drawn using polynomial regression. The *line* was drawn using linear regression. Despite the presence of a high affinity component, its density was too low to be detected by the fitting routine. Thus, the data did not fit a two-component model significantly better than a one-component model. However, the K_d (15 nM) for the Scatchard plot reflects the presence of the high affinity site. D, Scatchard plot of the 660 mM Ca^{2+} curve shown in A. Note the progressive diminution in the density of high affinity (+)-[^{3}H]pentazocine binding with increasing Ca^{2+} concentrations.

TABLE 4

Effects of selected cations on the saturation binding characteristics of (+)-[^{3}H]pentazocine

(+)-[^{3}H]Pentazocine (0.5–75 nM) was incubated with the indicated concentrations of Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Ni^{2+} and with a combination of Na^+, K^+, Ca^{2+}, and Mg^{2+} salts (Krebs-Ringer). K_d, equilibrium dissociation constant; B_{max}, maximal density of (+)-[^{3}H]pentazocine binding. The equilibrium binding constants were determined by nonlinear regression analysis of saturation binding data by fitting to a rectangular hyperbola model. When the two-component model fit the data significantly ($p < 0.05$) better than a one-component model, the K_{d_A}, K_{d_B}, B_{max_A}, and B_{max_B} values are presented. Each value represents the mean of three to 12 observations.

Treatment	One-site		Two-site			
	K_d	B_{max}	K_{d_A}	B_{max_A}	K_{d_B}	B_{max_B}
	nM	*fmol/mg of protein*	*nM*	*fmol/mg of protein*	*nM*	*fmol/mg of protein*
Control	3.7 ± 0.3	2400 ± 210				
Na^+, 120 mM	8.6 ± 0.4[a]	2600 ± 600				
Ca^{2+}						
4 mM			2.1 ± 0.6	650 ± 160	39 ± 10	1700 ± 170
30 mM	18 ± 3.3[a]	2200 ± 100				
660 mM	48 ± 3.1[a]	1300 ± 200[a]				
Mg^{2+}						
2 mM			2.6 ± 0.4	680 ± 160	64 ± 10	2100 ± 460
40 mM			2.3 ± 0.3	770 ± 79	43 ± 10	1600 ± 300
1.4 M	23 ± 5.8[a]	1100 ± 64[a]				
Zn^{2+}						
50 μM			2.2 ± 0.7	640 ± 180	33 ± 10	1540 ± 290
300 μM			1.8 ± 1.0	400 ± 22	21 ± 5.2	1600 ± 480
10 mM	15 ± 0.6[a]	1200 ± 54[a]				
Ni^{2+}, 400 μM			2.4 ± 0.2	860 ± 170	72 ± 17	1100 ± 45
Krebs-Ringer			1.9 ± 0.4	590 ± 140	30 ± 5.1	1800 ± 88

[a] Significantly different from control single-site constants, $p < 0.05$, by Scheffe's test after ANOVA.

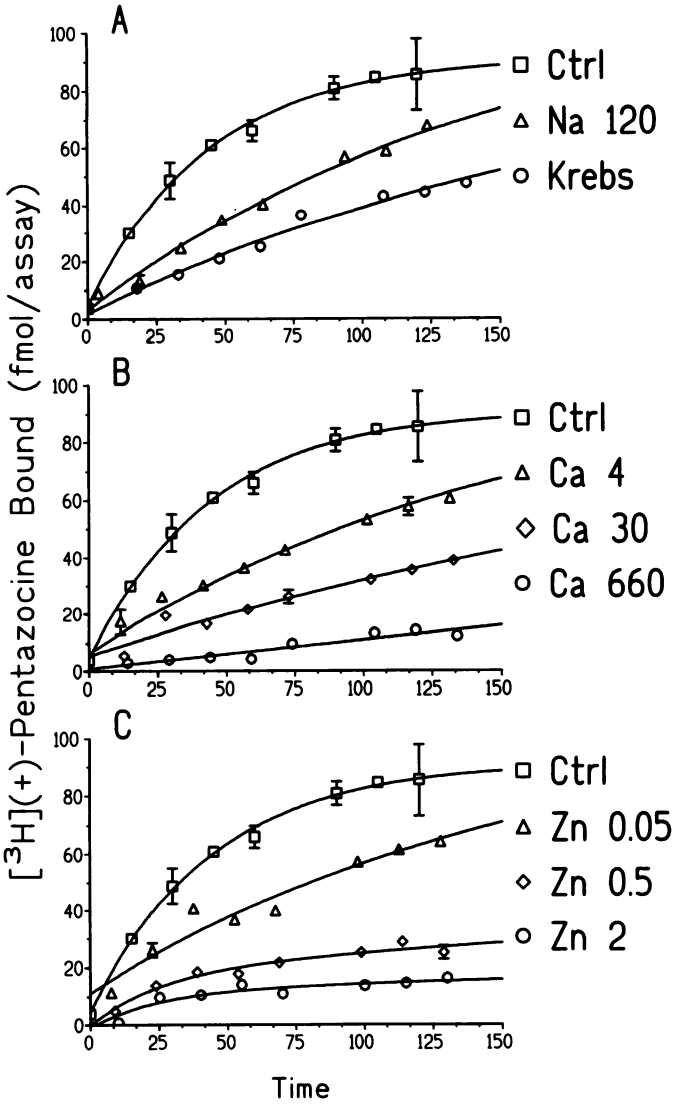

Fig. 3. Effects of selected cations on (+)-[^{3}H]pentazocine association with the σ receptor. *Curves* were fit to a single-component model using nonlinear regression. Each *curve* is a single representative experiment, with each *point* reproduced in triplicate. A, (+)-[^{3}H]Pentazocine binding in the presence of 120 mM Na^+ or Krebs-Ringer solution. B, Effects of 4–660 mM Ca^{2+} concentrations on (+)-[^{3}H]pentazocine binding. C, Effects of 0.05–2 mM concentrations of Zn^{2+} on (+)-[^{3}H]pentazocine binding. *Ctrl*, control.

TABLE 5

Effects of cations on the association and dissociation rates of (+)-[^{3}H]pentazocine

(+)-[^{3}H]Pentazocine (1, 2, or 5 nM) was incubated with the indicated concentrations of Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Ni^{2+} and with a combination of Na^+, K^+, Ca^{2+}, and Mg^{2+} salts (Krebs-Ringer). k_{obs}, observed association rate constant; k_{+1}, association rate constant; k_{-1}, dissociation rate constant. The k_{obs} and k_{-1} values were determined by nonlinear regression analysis of data from association and dissociation rate assays, respectively. Multiple-component analysis was not performed. The association rate constant (k_{+1}) was determined using the equation $k_{+1} = k_{obs} - k_{-1}/[L]$. Each value represents the mean of three or four observations at each radioligand concentration.

Treatment	Association rate ($t_{1/2}$)	k_{+1} ($\times 10^6$)	k_{-1} ($\times 10^{-3}$)
	min	*min*$^{-1}$	*min*$^{-1}$
Control	22 ± 2.0	7.7 ± 1.59	1.8 ± 0.19
Na^+, 120 mM	86 ± 12[a]	3.3 ± 0.18[a]	1.4 ± 0.28
Mg^{2+}			
2 mM	61 ± 15[a]	5.3 ± 0.78[b]	2.2 ± 0.12
40 mM	100 ± 37[a]	5.2 ± 0.15[b]	3.4 ± 2.0
1400 mM	69 ± 10[a]	4.5 ± 1.4[a]	0.75 ± 0.20
Ca^{2+}			
4 mM	44 ± 8.4[a]	3.9 ± 0.52[a]	2.6 ± 0.97
30 mM	87 ± 34[a]	2.6 ± 0.33[a]	2.8 ± 0.91
660 mM	200 ± 83[a]	2.9 ± 0.36[a]	2.8 ± 1.6
Zn^{2+}			
50 μM	48 ± 9.2[a]	2.3 ± 0.29[a]	2.7 ± 0.73
500 μM	53 ± 14[a]	2.8 ± 0.71[a]	4.2 ± 1.9
2 mM	66 ± 16[a]	1.3 ± 0.38[a]	3.5 ± 1.7
Ni^{2+}, 400 μM	130 ± 25[a]	2.0 ± 0.46[a]	3.2 ± 0.88
Krebs-Ringer	106 ± 24[a]	0.83 ± 0.26[a]	2.8 ± 0.25

[a] Significantly different from control values, $p < 0.05$, by Scheffe's test after ANOVA.
[b] $p < 0.05$, by Fisher's exact test after ANOVA.

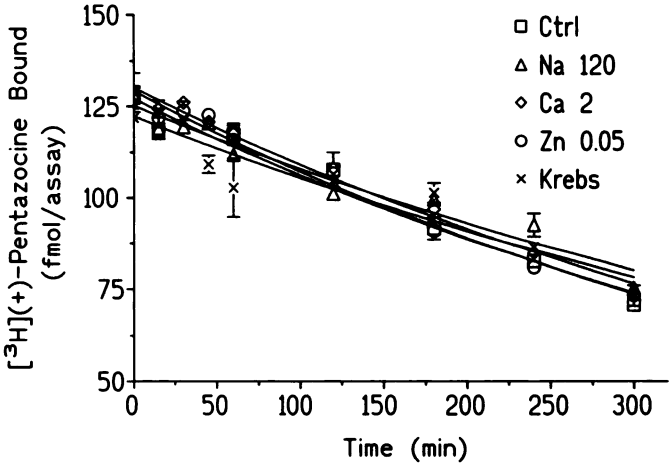

Fig. 4. Dissociation of (+)-[^{3}H]pentazocine binding from the σ receptor is unaffected by cations. The dissociation constants were determined from the slope of the *line*, using nonlinear regression techniques. The dissociation of (+)-[^{3}H]pentazocine was initiated, after 2 hr of incubation, by the addition of unlabeled (+)-pentazocine (10 μM) and the indicated cation. *Ctrl*, control.

be sufficiently low to allow a significant amount of ligand binding to these receptors to occur *in vivo*. Finally, the differential sensitivity of (+)-[^{3}H]pentazocine and [^{3}H]DTG binding to inhibition by cations and the biphasic inhibition of (+)-[^{3}H]pentazocine binding by di- and trivalent cations provide additional parameters for distinguishing σ_1 from σ_2 sites.

Saturation binding studies in the presence of physiologically relevant concentrations of Zn^{2+}, Ca^{2+}, and Mg^{2+} (38, 39) and in Krebs-Ringer buffer indicate that multiple (+)-[^{3}H]pentazocine binding sites exist *in vivo*. Analysis using a single-site model suggested that both mono- and divalent cations competitively inhibit (+)-[^{3}H]pentazocine binding. However, fitting the data to a two-site model revealed that (+)-[^{3}H]pentazocine binding in the presence of low concentrations of divalent cations consists of high and low affinity components. The high affinity (+)-[^{3}H]pentazocine binding site is very cation sensitive, because increasing concentrations of divalent cations selectively decreased its density. Only the highest concentrations of Ca^{2+}, Mg^{2+}, and Zn^{2+} tested significantly reduced the B_{max} for the low affinity (+)-[^{3}H]pentazocine binding site. Together, these results are consistent with a noncompetitive inhibition of (+)-[^{3}H]pentazocine binding.

The allosteric regulation of (+)-[^{3}H]pentazocine binding by divalent cations is also reflected in a significant decrease in the rate of association of (+)-[^{3}H]pentazocine with the σ_1 receptor in the presence of cations. However, the lack of effect of cations on the dissociation rate of (+)-[^{3}H]pentazocine is puzzling. This effect may result from a conformational change in the σ receptor after (+)-[^{3}H]pentazocine binding that decouples the ligand binding site from the cation binding site. This would

TABLE 6

Effects of guanine nucleotides on (+)-[^{3}H]pentazocine binding

A. Radioligand	Treatment	IC_{50}	n^a
[^{3}H]3-PPP	GTP	1.8 ± 0.1 mM	6
	GTP + Mg^{2+}	740 ± 32 μM	12
	GTPγS	160 ± 17 μM	3
	GTPγS + Mg^{2+}	160 ± 31 μM	9
(+)-[^{3}H]Pentazocine	GTP	10 ± 2.0 mM	4
	GTP + Mg^{2+}	14 ± 1.2 mM	11
	GTPγS	>600 μM	4
	GTPγS + Mg^{2+}	>600 μM	7

B. Cation	Control			+GTP		
	IC_{50}	Density	n	IC_{50}	Density	n
	mM	*% of total*		*mM*	*% of total*	
Na^+	74 ± 10		7	72 ± 13		7
Mg^{2+}						
A	2.0 ± 0.5	47 ± 5.9	6	2.9 ± 0.8	54 ± 4.9	11
B	730 ± 72			640 ± 110		
Ca^{2+}						
A	2.8 ± 0.6	59 ± 5.0	14	2.2 ± 0.6	65 ± 4.1	14
B	480 ± 110			500 ± 82		
Zn^{2+}						
A	0.044 ± 0.008	41 ± 6.1	7	0.035 ± 0.008	34 ± 9.0	11
B	1.5 ± 0.16			1.8 ± 0.25		

[a] *n*, number of experiments.

TABLE 7

Inhibition of specific (+)-[^{3}H]pentazocine binding to guinea pig cerebellum by various cation channel blockers

Pharmacological designations of these compounds are based on Refs. 46–48. Values are mean ± standard error of the mean.

Compound	IC_{50}	n^a
	nM	
L-type Ca^{2+} channel blockers		
Fluspirilene	840 ± 65.0	3
MDL 12330A	861 ± 27.4	4
Verapamil	2,390 ± 70.0	3
Diltiazem	6,220 ± 810	3
Nifedipine	>10,000	3
T-type Ca^{2+} channel blocker		
Amiloride	>10,000	3
N-type Ca^{2+} channel blocker		
ω-Conotoxin	26,500 ± 4,870	3
Ca^{2+} channel activator		
Bay K-8644	1,160 ± 423	3
Intracellular Ca^{2+} channel blocker		
TMB-8	98.7 ± 25.6	5
Receptor-gated Ca^{2+} channel blockers		
Econazole	>10,000	4
SKF 96365	10,700 ± 700	3
Nonselective Na^+/Ca^{2+} channel blockers		
Proadifen	12 ± 1.2	3
Prenylamine	128 ± 11.2	6
Amiodarone	146 ± 26.5	4
Cinnarizine	162 ± 28.8	6
Hydroxyzine	192 ± 17.7	4
Pimozide	1,000 ± 60.0	7
Bepridil	1,810 ± 620	4
Propranolol	4,360 ± 186	3
Na^+ channel blockers		
Tetracaine	61.7 ± 13.0	3
Diphenhydramine	1,700 ± 300	3
Cocaine	>10,000	3
Procainamide	>10,000	3
Tetrodotoxin	>30,000	3

[a] *n*, number of experiments.

reduce the influence of cations upon ligand binding until the ligand dissociates from its binding site.

The development of multiple affinity sites for (+)-[^{3}H]pentazocine binding in the presence of divalent cations may represent different states of the σ_1 receptor rather than the interconversion of σ_1 to σ_2 subtypes. Such an interconversion is unlikely for the following reasons. (+)-[^{3}H]Pentazocine binds with high affinity (K_d = 4 nM) and selectivity to the σ_1 subtype, while showing low affinity (K_i = 200–400 nM) for the σ_2 receptor (21, 25, 29). Thus, (+)-[^{3}H]pentazocine binding to σ_2 receptors is not likely to be detected under the conditions of this binding assay and is not close to the K_d value for the low affinity component of (+)-[^{3}H]pentazocine binding reported here. Furthermore, if (+)-[^{3}H]pentazocine bound to a mixed population of σ_1 and σ_2 receptors, low concentrations of divalent cations would tend to increase B_{max_B}. However, no decrease in the combined $B_{max_{A,B}}$ for (+)-[^{3}H]pentazocine was observed at all but the highest cation concentrations. Finally, σ_2 receptor density would be increased by cations. This is not the case, inasmuch as divalent cations inhibited [^{3}H]DTG binding to σ_2 receptors by decreasing the B_{max}.

The significance of the multiple affinity states is unclear. The data may reflect the simultaneous binding of (+)-[^{3}H]pentazocine to two sites, one of which is highly cation sensitive, that normally are located in different subcellular regions. However, (+)-[^{3}H]pentazocine binding is located primarily in endoplasmic reticulum (36), and the two sites appear to share pharmacological characteristics, such as their inhibition by nonselective cation channel blockers and lack of coupling to G proteins. Biphasic saturation isotherms are also indicative of the formation of ternary complexes, such as G protein coupling to the σ_1 receptor. In contrast to previous reports of the inhibition of (+)-[^{3}H]3-PPP and (+)-[^{3}H]SKF 10047 binding by GTP, Gpp(NH)p, or PTX (26, 28), (+)-[^{3}H]pentazocine binding was not consistently altered by guanine nucleotides or membrane treatment with CTX or PTX. Furthermore, the effects of cations on (+)-[^{3}H]pentazocine binding were not consistently modified by guanine nucleotides. This lack of effect of guanine nucleotides suggests that (+)-pentazocine acts as an antagonist and is incapable of causing the changes in σ_1 receptor conformation required for G protein coupling. Alternatively, these observations may provide evidence for the presence of several σ receptor subtypes, one of which is not coupled to G proteins (21, 29).

The functional significance of cation modulation of ligand binding to σ receptor subtypes is not known. The ability of cations to modulate ligand binding to σ receptors may have only pharmacological relevance, as in the enhancement of agonist or antagonist binding to opiate receptors by monovalent or divalent cations (40, 41). However, the potent inhibition of (+)-[^{3}H]pentazocine binding by di- and trivalent cations may reflect the association of the σ_1 (and possibly the σ_2) receptor with a cation channel. This hypothesis is further supported by the ability of several nonselective cation channel blockers (e.g., cinnarizine, prenylamine, proadifen, and tetracaine) to inhibit (+)-[^{3}H]pentazocine binding. The association of σ receptors with the regulation of cation biochemistry would be consistent with observations of σ receptor ligands modulating such calcium-dependent functions as the inhibition of catecholamine release from adrenal chromaffin cells (19), suppression of epileptogenesis (42), modulation of NMDA-induced neuronal activation (43, 44), and protection against ischemic cytotoxicity (45). Future investigations of σ receptor functions may center on the role of these receptors in the modulation of calcium currents or the regulation of intracellular calcium concentration.

References

1. Su, T.-P. Psychotomimetic opiate binding: specific binding of [3H]SKF 10047 to etorphine-inaccessible sites in guinea pig brain. *Eur. J. Pharmacol.* **75**:81–82 (1981).
2. Su, T.-P. Evidence for σ opioid binding: binding of [3H]SKF 10047 to etorphine inaccessible sites in guinea pig brain. *J. Pharmacol. Exp. Ther.* **223**:284–290 (1982).
3. Tam, S. W., and L. Cook. σ opiates and certain antipsychotic drugs mutually inhibit (+)-[3H]SKF 10047 and (+)-[3H]haloperidol binding in guinea pig brain membranes. *Proc. Natl. Acad. Sci. USA* **81**:5618–5621 (1984).
4. Samovilova, N. N., L. V. Nagornaya, and V. A. Vinogradov. (+)-[3H]SKF 10047 binding sites in rat liver. *Eur. J. Pharmacol.* **147**:259–264 (1988).
5. Su, T.-P., S. E. Schell, F. Y. Ford-Rice, and E. D. London. Correlation of inhibitory potencies of putative antagonists for σ receptors in brain and spleen. *Eur. J. Pharmacol.* **148**:467–470 (1988).
6. Tam, S. W. Naloxone-inaccessible *sigma* receptor in rat central nervous system. *Proc. Natl. Acad. Sci. USA* **80**:6703–6707 (1983).
7. Tam, S. W. (+) [3H]SKF10047, (+) [3H]ethylketocyclazocine, μ, δ, κ, and phencyclidine binding sites in guinea pig brain membranes. *Eur. J. Pharmacol.* **109**:33–41 (1985).
8. Gundlach, A. L., B. L. Largent, and S. H. Snyder. Autoradiographic localization of σ receptor binding sites in guinea pig and rat central nervous system with (+)-[3H]-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine. *J. Neurosci.* **6**:1757–1770 (1986).
9. Manallack, D. T., and P. M. Beart. Quantitative conformational analyses predict distinct receptor sites for PCP-like and σ drugs. *Eur. J. Pharmacol.* **144**:231–235 (1987).
10. de Costa, B. R., W. D. Bowen, S. B. Hellewell, J. M. Walker, A. Thurkauf, A. E. Jacobsen, and K. C. Rice. Synthesis and evaluation of optically pure [3H] (+)-pentazocine, a highly potent and selective radioligand for *sigma* receptors. *FEBS Lett.* **251**:53–58 (1989).
11. de Costa, B. R., W. D. Bowen, S. B. Hellewell, J. M. Walker, A. Thurkauf, A. E. Jacobsen, and K. C. Rice. Synthesis and evaluation of optically pure [3H](+)-pentazocine, a highly potent and selective radioligand for σ receptors. *Prog. Clin. Biol. Res.* 117–120 (1990).
12. Bowen, W. D., B. R. de Costa, S. B. Hellewell, A. Thurkauf, J. M. Walker, and K. C. Rice. Characterization of [3H] (+)-pentazocine, a highly selective *sigma* ligand. *Prog. Clin. Biol. Res.* 117–120 (1990).
13. Bowen, W. D., B. N. Kirschner, A. H. Newman, and K. C. Rice. σ receptors negatively modulate agonist-stimulated phosphoinositide metabolism in rat brain. *Eur. J. Pharmacol.* **149**:399–400 (1988).
14. Brog, J. S., and M. C. Beingeld. Inhibition of carbachol-induced inositol phosphate accumulation by phencyclidine, phencyclidine-like ligands and *sigma* agonists involves blockade of the muscarinic cholinergic receptor: a novel dioxadrol-preferring interaction. *J. Pharmacol. Exp. Ther.* **254**:952–956 (1990).
15. Lodge, D., J. A. Aram, J. Church, S. N. Davies, E. Fletcher, and D. Martin. Electrophysiological studies of the interaction between phencyclidine/*sigma* receptor agonists and excitatory amino acid neurotransmission on central mammalian neurones, in *Sigma and Phencyclidine-Like Compounds as Molecular Probes in Biology* (E. F. Domino and J.-M. Kamenka, eds.). NPP Books, Ann Arbor, MI, 239–244 (1988).
16. Malouf, A. T., E. S. Swearengen, S. Mailheau, and C. Chavkin. Electrophysiological actions of *sigma* ligands in the *in vitro* hippocampus, in *Sigma and Phencyclidine-Like Compounds as Molecular Probes in Biology* (E. F. Domino and J.-M. Kamenka, eds.). NPP Books, Ann Arbor, MI, 229–238 (1988).
17. Wu, X.-Z., J. A. Bell, C. E. Spivak, E. D. London, and T.-P. Su. Electrophysiological and binding studies on intact NCB-20 cells suggest the presence of a low affinity *sigma* receptor. *J. Pharmacol. Exp. Ther.* **257**:351–359 (1991).
18. Riviere, P. J., X. Pascaud, J. L. Junien, and F. Porreca. Neuropeptide Y and JO 1784, a selective *sigma* ligand, alter intestinal ion transport through a common, haloperidol-sensitive site. *Eur. J. Pharmacol.* **187**:557–559 (1990).
19. Paul, I. A., E. Rojas, M. B. H. Youdim, B. De Costa, P. Skolnick, H. B. Pollard, and G. A. J. Kuijpers. *Sigma* receptor modulation of nicotinic responses in adrenal chromaffin cells, in *Multiple Sigma and PCP Receptor Ligands: Mechanisms for Neuromodulation and Protection* (E. F. Domino and J. M. Kamenka, eds.). NPP Books, Ann Arbor, MI, 527–542 (1992).
20. Largent, B. L., H. Wikstrom, A. L. Gundlach, and S. H. Snyder. Structural determinants of *sigma* receptor affinity. *Mol. Pharmacol.* **32**:772–784 (1987).
21. Walker, J. M., W. D. Bowen, F. O. Walker, R. R. Matsumoto, B. de Costa, and K. C. Rice. *Sigma* receptors: biology and function. *Pharmacol. Rev.* **42**:355–402 (1991).
22. Martin, W. R., C. G. Eades, J. A. Thompson, R. E. Huppler, and P. E. Gilbert. The effects of morphine and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J. Pharmacol. Exp. Ther.* **197**:517–532 (1976).
23. Quirion, R., R. Chicheportiche, P. C. Contreras, K. M. Johnson, D. Lodge, S. W. Tam, J. H. Woods, and S. R. Zukin. Classification and nomenclature of phencyclidine and *sigma* receptor sites. *Trends Neurosci.* **10**:444–446 (1987).
24. Fox, P. K., J. H. Connick, G. M. Hanlon, L. France, and C. D. Nicholson. The lack of utility of the rat vas deferens as a functional bioassay for σ ligands. *Eur. J. Pharmacol.* **193**:139–143 (1991).
25. Rothman, R. B., A. Reid, A. Mahboubi, C.-H. Kim, B. R. de Costa, A. E. Jacobsen, and K. C. Rice. Labeling by [3H]1,3-di(2-tolyl)guanidine of two high affinity binding sites in guinea pig brain: evidence for allosteric regulation by calcium channel antagonists and pseudoallosteric modulation by σ ligands. *Mol. Pharmacol.* **39**:222–232 (1991).
26. Beart, P. M., R. D. O'Shea, and D. T. Manallack. Regulation of σ receptors: high and low affinity agonist states, GTP shifts and up-regulation by rimcazole and 1,3-di(2-tolyl)guanidine. *J. Neurochem.* **53**:779–788 (1989).
27. Itzhak, Y., and M. Khouri. Regulation of the binding of σ- and phencyclidine (PCP)-receptor ligands in rat brain membranes by guanine nucleotides and ions. *Neurosci. Lett.* **85**:147–152 (1988).
28. Itzhak, Y. Multiple affinity binding states of the σ receptor: effect of GTP-binding protein-modifying agents. *Mol. Pharmacol.* **36**:512–517 (1989).
29. Quirion, R., W. D. Bowen, Y. Itzhak, J. L. Junien, J. M. Musacchio, R. B. Rothman, T. P. Su, S. W. Tam, and D. P. Taylor. A proposal for the classification of *sigma* binding sites. *Trends Pharmacol. Sci.* **13**:85–86 (1992).
30. Vargas, H. M., and R. N. Pechnick. Binding affinity and antimuscarinic activity of σ and phencyclidine receptor ligands. *Eur. J. Pharmacol.* **195**:151–156 (1991).
31. Dohanich, G. P., A. E. Johnson, B. Nock, B. S. McEwen, and H. H. Feder. Distribution of cholinergic muscarinic binding sites in guinea pig brain as determined by *in vitro* autoradiography of [3H]*N*-methylscopolamine binding. *Eur. J. Pharmacol.* **119**:9–16 (1985).
32. Camps, M., P. H. Kelly, and J. M. Palacios. Autoradiographic localization of dopamine D1 and D2 receptors in the brain of several mammalian species. *J. Neural Transm. Gen. Sect.* **80**:105–127 (1990).
33. Maragos, W. F., J. B. Penney, and A. B. Young. Anatomic correlation of NMDA and [3H]TCP-labeled receptors in rat brain. *J. Neurosci.* **8**:493–501 (1988).
34. McLean, S., and E. Weber. Autoradiographic visualization of haloperidol-sensitive *sigma* receptors in guinea pig brain. *Neuroscience* **25**:259–269 (1988).
35. Koe, B. K., C. A. Burkhart, and L. A. Lebel. (+)-[3H]3-(3-Hydroxyphenyl)-*N*-(1-propyl)-piperidine binding to σ receptors in mouse brain *in vivo*. *Eur. J. Pharmacol.* **161**:263–266 (1989).
36. Basile, A. S., I. A. Paul, and B. de Costa. Differential effects of cytochrome P-450 induction on σ receptor ligand binding. *Eur. J. Pharmacol.* **227**:95–98 (1992).
37. McCann, D. J., R. A. Rabin, S. Rens-Domiano, and J. C. Winter. Phencyclidine/SKF 10047 binding sites: evaluation of function. *Pharmacol. Biochem. Behav.* **32**:87–94 (1989).
38. Howell, G. A., M. G. Welch, and C. J. Frederickson. Stimulation-induced uptake and release of zinc in hippocampal slices. *Nature (Lond.)* **308**:736–738 (1984).
39. Hille, B., and W. A. Catterall. Electrical excitability and ionic channels, in *Basic Neurochemistry* (G. J. Siegel, B. W. Agranoff, R. W. Albers, and P. B. Molinoff, eds.), Ed. 4. Raven Press, New York, 71–90 (1989).
40. Pert, C. B., and S. H. Snyder. Properties of opiate receptor binding in rat brain. *Proc. Natl. Acad. Sci. USA* **70**:2243–2247 (1973).
41. Simon, E. J., J. M. Hiller, J. Groth, and I. Edelman. Further properties of stereospecific opiate binding sites in rat brain: on the nature of the sodium effect. *J. Pharmacol. Exp. Ther.* **192**:531–537 (1975).
42. Aram, J. A., D. Martin, M. Tomczyk, S. Zeman, J. Millar, G. Pohler, and D. Lodge. Neocortical epileptogenesis *in vitro*: studies with *N*-methyl-D-aspartate, phencyclidine, *sigma* and dextromethorphan receptor ligands. *J. Pharmacol. Exp. Ther.* **248**:320–328 (1989).
43. Iyengar, S., V. M. Dilworth, S. J. Mick, P. C. Contreras, J. B. Monahan, T. S. Rao, and P. L. Wood. *Sigma* receptors modulate both A9 and A10 dopaminergic neurons in the rat brain: functional interaction with NMDA receptors. *Brain Res.* **524**:322–326 (1990).
44. Pontecorvo, M. J., E. W. Karbon, S. Goode, D. B. Clissold, S. A. Borosky, R. J. Patch, and J. W. Ferkany. Possible cerebroprotective and *in vivo* NMDA antagonist activities of *sigma* agents. *Brain Res. Bull.* **26**:461–465 (1991).
45. Lobner, D., and P. Lipton. *Sigma*-ligands and non-competitive NMDA antagonists inhibit glutamate release during cerebral ischemia. *Neurosci. Lett.* **117**:169–174 (1990).
46. Godfraind, T., R. Miller, and M. Wibo. Calcium antagonism and calcium entry blockade. *Pharmacol. Rev.* **38**:321–416 (1986).
47. Seiler, S. M., A. J. Arnold, and H. C. Stanton. Inhibitors of inositol triphosphate-induced Ca^{++} release from isolated platelet membrane vesicles. *Biochem. Pharmacol.* **36**:3331–3337 (1987).

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