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Zinc Modulation of Drug Binding, Cocaine Affinity States, and Dopamine Uptake on the Dopamine Uptake Complex

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SUMMARY

Zinc plays an important role in synaptic function in the brain. Zinc is present in synaptic vesicles, is released with neuronal activity, and provides modulation of different neurotransmitter systems. Zinc altered characteristics of the dopamine uptake complex in rat caudate-putamen. The density of the dopamine uptake complex labeled with [^{3}H]GBR 12935 (0.25 nM) was increased by up to 215% in the presence of zinc (10 μM), compared with control. Augmentation of binding occurred with concentrations of zinc from 0.5 to 100 μM. This effect on the dopamine uptake complex was due to a 65% decrease in the equilibrium dissociation constant for [^{3}H]GBR 12935 without a significant change in the total number of binding sites. The presence of exogenous zinc (50 μM) increased the percentage of high affinity sites for cocaine from 73 ± 4% to 90 ± 1% and decreased the high affinity constant from 2250 ± 880 nM to 890 ± 150 nM. Similar effects of zinc occurred with the cocaine congener 2β-carbomethoxy-3β-(fluorophenyl)tropane. Zinc inhibited dopamine uptake into synaptosomes at concentrations from 0.5 to 100 μM. Zinc (0.5 and 1 μM) augmented cocaine inhibition of uptake by 58–79%. Modulation of drug binding to the dopamine uptake complex is another role for zinc in the central nervous system. Sulfhydryl oxidation of the dopamine uptake complex by certain metal cations (Cu^{2+}, Hg^{2+}, and Cd^{2+}) or *N*-methylmaleimide reduced binding of [^{3}H]GBR 12935 to the dopamine uptake complex. The sulfhydryl oxidation by *N*-methylmaleimide was blocked by coincubation with dithiothreitol and dopamine uptake blockers. These data support the existence of a sulfhydryl group involved in binding of uptake blockers to the dopamine uptake complex.

Our understanding of zinc as a neuromodulator in the CNS is growing (1, 2). Support for zinc as a neuromodulator comes from studies that show it has a unique distribution in the CNS of mammals, including the neostriatum (1–4), is contained in synaptic vesicles, and is released with neuronal activity (5–8). Zinc has recently been demonstrated to have an effect on amino acid neurotransmission because of its neuronal and synaptic localization and its effects at excitatory and inhibitory amino acid receptor complexes (9–12). Zinc influences the DA system by partial inhibition of both DA uptake and ligand binding to the DA D-2 receptor (13, 14). A specific biochemical interaction between zinc and the DAUC has not been reported and is the major topic of this report.

Direct saturation binding or competition studies using cocaine or cocaine congeners have demonstrated two affinity states for cocaine on the DAUC (15–20). These two affinity states may represent two proteins having different amino acid sequences, a single protein undergoing post-translational modification, or a single protein having interconvertible states modulated by a soluble substance. Modulation of affinity states *in vitro* would support the last model explaining the nature of the two sites.

Cocaine exerts its reinforcing properties by interaction with the DAUC (21). Acute and chronic cocaine use, as well as withdrawal from cocaine, results in a variety of behavioral effects in animals (22, 23). These effects include tolerance and sensitization. Humans demonstrate unique behavioral effects in response to cocaine exposure and withdrawal (24). These effects contribute to the addictive nature of cocaine and to cocaine recidivism (25). Neurobiological correlates of behavioral responses to cocaine are unclear but may involve the DAUC, DA receptors (22), or other neurotransmitter systems. Cocaine affinity states on the DAUC may play an important role in mediating different behavioral responses to cocaine.

This study describes the effect of zinc and other divalent cations on binding properties and uptake of the DAUC. These findings support the presence of an allosteric effector site for zinc on the DAUC that influences both binding and DA uptake.

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ABBREVIATIONS: B_{max}, maximal number of binding sites; CNS, central nervous system; CPu, caudate-putamen; DA, dopamine; DAUC, dopamine uptake complex; DTT, dithiothreitol; K_d, equilibrium dissociation constant; K_H and K_L, high and low affinity equilibrium dissociation constants, respectively; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-methylmaleimide; NMDA, *N*-methyl-D-aspartate; B_H and B_L, high and low affinity binding sites, respectively; WIN 35,428, 2β-carbomethoxy-3β-(fluorophenyl)tropane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ANOVA, analysis of variance.

Materials and Methods

Tissue procurement. Rodents were acquired and cared for in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Tissue used for autoradiography was obtained by rapid decapitation of adult male Sprague-Dawley rats (180–220 g). Brains were frozen by immersion in isopentane at −30° for 5–10 sec followed by several minutes in powdered dry ice and were stored at −70° until sectioning. Brains were mounted on tissue pedestals with Lipshaw embedding matrix and warmed to −18° to −20°; coronal sections (10 μm) were cut on a cryostat microtome and thaw-mounted onto gelatin-coated slides. Slides were dehydrated at 25° and then frozen at −30° until used in assays.

Tissue used for DA uptake was obtained by decapitation after CO_2 exposure. A coronal slice (1.7 mm) through the CPu was obtained using a tissue slicer (Stoelting Co., Wood Dale, IL) and the CPu was blocked on ice, weighed, and immediately homogenized.

Binding conditions. Binding conditions were similar to those previously described (17–19). One [^{3}H]GBR 12935 binding buffer was a 50 mM sodium phosphate buffer (pH 7.5), with a final Na^+ concentration of 120 mM obtained using NaCl, 0.001% ascorbate, 0.025% fatty acid-free bovine serum albumin, and 0.75 μM *trans*-flupentixol. Competition and saturation studies used this buffer to allow comparison with previous work. Nine other buffers were tested to select a buffer that would not precipitate with certain divalent cations. A 25 mM MOPS buffer (pH 7.5) containing 120 mM NaCl, 0.001% ascorbate, 0.75 μM *trans*-flupentixol, and 0.025% fatty acid-free bovine serum albumin was used for studies with varying concentrations of different divalent cations and for saturation studies. Slides used for autoradiography were warmed to 2–3° for 30 min, incubated with [^{3}H]GBR 12935 (0.25 nM for single-concentration studies or 0.10–10 nM for saturation studies) at 2–3° for 30 hr, washed in fresh buffer at 2–3° for 2 hr, and then dried with a stream of cool air. Mazindol (25 μM) was used to determine nonspecific binding in each experiment. Nonspecific binding was measured for each ion concentration and with each buffer tested. All values reported represent specific binding. Two slides, each containing one 10-μm-thick section through the CPu, were incubated in a glass vial containing 10 ml of buffer with ligand. Aliquots of the incubation buffer were sampled at the beginning and end of each experiment to ensure that the free ligand concentration did not decline by >10%.

For experiments using NEM, tissue sections were preincubated in buffer with or without mazindol (25 μM), cocaine (100 μM), or DTT (10 mM) for 120 min. Slides were then washed in buffer for 5 min, incubated in buffer with NEM (5–100 mM) in the absence or presence of mazindol (25 μM), cocaine (100 μM), or DTT (10 mM) for 120 min, washed for 2 × 10 min in buffer, and finally placed in the standard assay for the DAUC as described above.

Autoradiography and densitometry. Dried slides were placed in a X-ray cassette with ^{14}C plastic standards previously calibrated with ^{3}H brain paste sections (26) and were exposed to LKB Ultrofilm ^{3}H at 25° for 10–21 days. The LKB Ultrofilm ^{3}H was developed in Kodak D19 for 4 min at room temperature, stopped in 1.5% acetic acid, fixed in Kodak Rapid Fix for 3.5 min, and washed in tap water for 20 min. Binding data were obtained from the rat CPu using densitometry (M1 version 3.61; MCID BRS, Imaging Research, Inc.) for all studies. The dorsal striatum (CPu) from both hemispheres was outlined in each section using a mouse-driven cursor. Duplicate sections were used to measure total and nonspecific binding, and single sections were used for each condition with varying concentrations of cocaine or WIN 35,428. Subtracting the amount bound in the presence of 25 μM mazindol (nonspecific binding) from the amount bound in the absence of mazindol (total binding) produced the specific binding. Two animals were used in each experiment and three to six independent experiments were done for each type of condition.

DA uptake. CPu tissue was immediately homogenized in 20 volumes of ice-cold 0.32 M sucrose, 50 mM Tris-HEPES (pH 7.4), using a Teflon-glass homogenizer (clearance, 0.15 mm), with 14 up-and-down rotating strokes by hand. The homogenate was centrifuged at 900 × *g* for 10 min. The supernatant was then centrifuged at 17,000 × *g* for 20 min. The pellet was resuspended in 20 volumes of ice-cold incubation buffer and mixed by vortexing. The incubation buffer consisted of 10 mM Tris-HEPES (pH 7.4), 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 0.25 mM ascorbate, and 15 μM pargyline. The reaction volume was 0.5 ml. [^{3}H]DA was used at a concentration of 10 nM and unlabeled DA (90 nM) was added to achieve a final concentration of 100 nM for all experiments except saturation studies. Other drugs and ions were prepared in the incubation buffer and kept on ice until added to the reaction mixture. The reaction mixture was kept on ice until a 5-min prewarming at 37° followed by incubation for 6 min (except for kinetic studies) at 37°. Uptake was terminated by the addition of 3 ml of ice-cold normal saline; tubes were placed on ice until filtration. The synaptosomes were rapidly filtered (Whatman GF/C filter paper) using a 48-probe harvester (Brandel Inc., Gaithersburg, MD), and washed twice with 3 ml of cold saline. Filters were counted using a liquid scintillation counter (LKB Rackbeta) using 5 ml of Ecoscint H (National Diagnostics) cocktail. Counting efficiency was corrected for each sample and averaged about 40%. Total uptake occurring at 37° was corrected by subtracting uptake occurring at 0°, and temperature-specific uptake averaged 94%. Uptake was proportional to tissue concentration and DA concentration and was time dependent (data not shown). Protein was measured using the Bio-Rad protein assay (Bio-Rad, Richmond, CA).

Data and statistical analysis. The CPu was used for all determinations of density. Competition experiments were analyzed using both linear and nonlinear methods. Linear transformations were used to determine Hill coefficients (27) and inhibitory constants. One- and two-site nonlinear analyses were performed using a computer-assisted least-squares iterative modeling program (Lundon Software, Inc., Cleveland, OH). All curves were first analyzed as a single-site fit. Each cocaine and WIN 35,428 competition curve was best modeled as a two-site fit based on a significant reduction in the sum of squares error (compared with the one-site fit) as determined by an *F* test ($p < 0.05$) and an insignificant runs test ($p > 0.05$) (28, 29). Competition models were constrained by having the K_d for [^{3}H]GBR 12935 fixed to a single value that was determined by independent saturation experiments.

Single-concentration ion effects were statistically analyzed using one-way ANOVA of the ratio of cation effect to control binding, followed by individual *t* tests for each cation using the mean-square error from the one-way ANOVA (SYSTAT, Evanston, IL). Corrections for multiple comparisons were made using the Bonferroni correction for nine *post hoc* tests, with $p < \alpha/n$ ($\alpha = 0.05$, $n = 9$) or $p < 0.006$.

Binding parameters from saturation experiments were statistically analyzed using one-way ANOVA followed by individual contrasts comparing the change in B_{max} and K_d in the presence of each cation with control values obtained in the absence of added cations (SYSTAT). Corrections for multiple comparisons were made using the Bonferroni correction for eight *post hoc* tests, with $p < \alpha/n$ ($\alpha = 0.05$, $n = 8$) or $p < 0.006$.

Competition parameters were analyzed using one-way ANOVA (SYSTAT) comparing each parameter mean in the absence and presence of zinc for the two competitors. Bonferroni correction for three *post hoc* comparisons (for each competitor) using $p < \alpha/n$ ($\alpha = 0.05$, $n = 3$) or $p < 0.017$ was used.

Materials. [^{3}H]GBR 12935 (specific activity, 13–45 Ci/mmol) and [^{3}H]DA (specific activity 26.7 Ci/mmol) were obtained from New England Nuclear/Du Pont (Wilmington, DE). *trans*-Flupentixol was a gift from Dr. John Hyttel of H. Lundbeck and Co. (Copenhagen, Denmark). Cocaine, fatty acid-free bovine serum albumin, DA, NEM, DTT, pargyline, all cations, and buffers were obtained from Sigma (St. Louis, MO). WIN 35,428 was obtained from Research Biochemicals, Inc. (Natick, MA). Mazindol was a gift from Sandoz Pharmaceuticals (East Hanover, NJ).

Results

Buffer studies. Although sodium phosphate was shown to be a good buffer for measuring the density of the DAUC (18), it could not be used for some studies involving divalent cations. A single concentration of [^{3}H]GBR 12935 (0.25 nM) was used to measure the density of the DAUC in the presence of 10 different buffers. Other components of the buffer (pH, [Na^+], [Cl^-], [albumin], and [ascorbate]) were identical. A sodium phosphate buffer was again found to produce the highest density at a single concentration (Fig. 1). All other buffers resulted in variably decreased density, with a MOPS buffer having the least decrease in density. The MOPS buffer was subsequently used for saturation and divalent cation studies. The MOPS buffer resulted in B_{max} (11.6 ± 1.0 pmol/mg of protein) and K_d (4.3 ± 0.9 nM) values that differed from those obtained with the phosphate buffer (B_{max} = 7.0 ± 1.1 pmol/mg of protein and K_d = 2.1 ± 0.2 nM).

Cation studies. Tissue sections containing the CPu were coincubated with nine divalent cations (100 μM) that have known effects on other neurotransmitter systems in the MOPS buffer (Fig. 2). Cations with high reactivity to sulfhydryl groups resulted in a significant decline in the density of the DAUC in a rank order that correlated with their sulfhydryl reactivity (Hg^{2+} > Cu^{2+} > Cd^{2+}) (30). The effect of Cd^{2+} was due to a decrease in B_{max} and not due to a change in the K_d (Fig. 3; Table 1). Three cations (Mg^{2+}, Mn^{2+}, and Ca^{2+}), at a concentration of 100 μM, had no significant effect on the density of the DAUC (Fig. 2).

Three transition metal cations (Co^{2+}, Ni^{2+}, and Zn^{2+}) at a concentration of 100 μM resulted in increased binding (170–243%), compared with control (Fig. 2). The effect of each transition metal ion was to decrease significantly the K_d of the DAUC for [^{3}H]GBR 12935 without altering the B_{max} (Fig. 3; Table 1). The effect of zinc on the K_d of the DAUC for [^{3}H]GBR 12935 was not unique to the MOPS buffer. Zinc also significantly decreased the K_d (1.0 ± 0.1 nM) without significantly changing the B_{max} (6.3 ± 0.2 pmol/mg of protein) of the DAUC in the phosphate buffer. The magnitude of the decrease in the K_d was similar among the three transition metal ions (56–65%) and for zinc using the two different buffers (52–65%). The concentration of each transition metal cation varied for this effect, with zinc having the highest apparent affinity (Table 1). Enhanced [^{3}H]GBR 12935 binding was seen with zinc concentrations from 0.5 to 100 μM; higher concentrations of zinc (500–1000 μM) resulted in decreased binding (Fig. 4). None of the effects described were related to the anion of the salt, because chloride and sulfate did not have selective effects by themselves (Fig. 2).

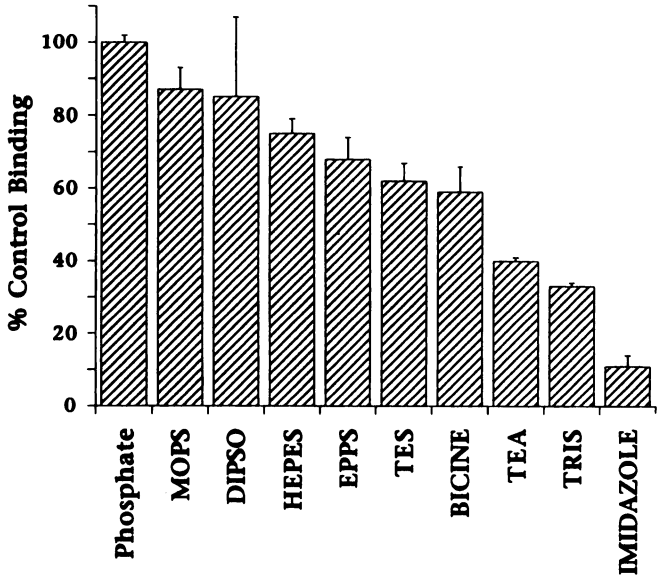

Fig. 1. Bar chart of the effect of different buffers on binding to the DAUC in rat CPu at a single concentration of [^{3}H]GBR 12935 (0.25 nM). All other components of the buffer were similar. Values (mean and standard error) are percentage of control value (sodium phosphate buffer). Each buffer tested resulted in a variable decrease, compared with sodium phosphate, with MOPS having the least decrement. *DIPSO*, 3-[*N*,*N*-bis-(2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; *EPPS*, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); *TES*, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; *TEA*, triethanolamine.

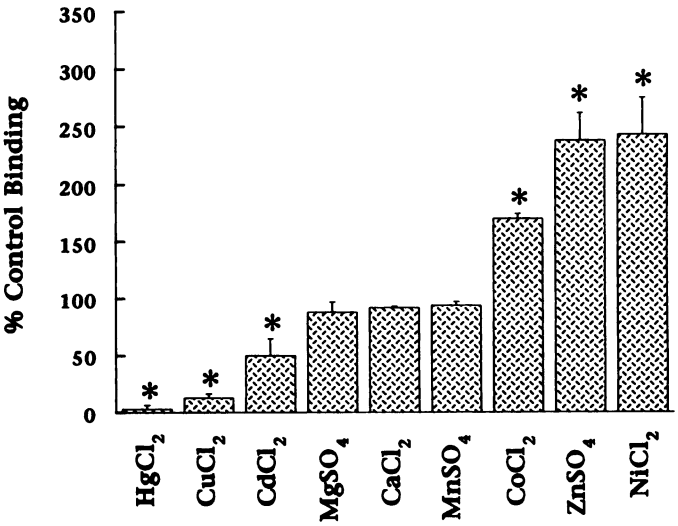

Fig. 2. Bar chart of cation effects on [^{3}H]GBR 12935 binding in rat CPu. The effect of 100 μM divalent cation on the binding of [^{3}H]GBR 12935 to the DAUC is shown. Values represent the percentage of binding in the presence of added cation (mean ± standard error), compared with binding in the absence of added cation, from two to four independent experiments. These values do not represent the maximal effect or lowest effective concentration of the different cations but do indicate the relative effect of each cation at 100 μM. *, Cations significantly different from control (Bonferroni correction for nine *post hoc* tests with $p < \alpha/n$, α = 0.05, n = 9, or $p < 0.006$).

Cocaine competition studies. Cocaine and the potent cocaine congener WIN 35,428 displaced binding of [^{3}H]GBR 12935 at the DAUC with two affinities (Fig. 5; Table 2). In the absence of exogenous zinc the proportion of R_H ranged from 73 ± 4% (cocaine) to 84 ± 1% (WIN 35,428). In the presence of zinc (50 μM) the proportion of R_H increased significantly to 90 ± 2% (cocaine) or 93 ± 1% (WIN 35,428). The K_H for cocaine and WIN 35,428 decreased in the presence of zinc. For cocaine, the K_H decreased significantly from 2,250 ± 880 nM in the

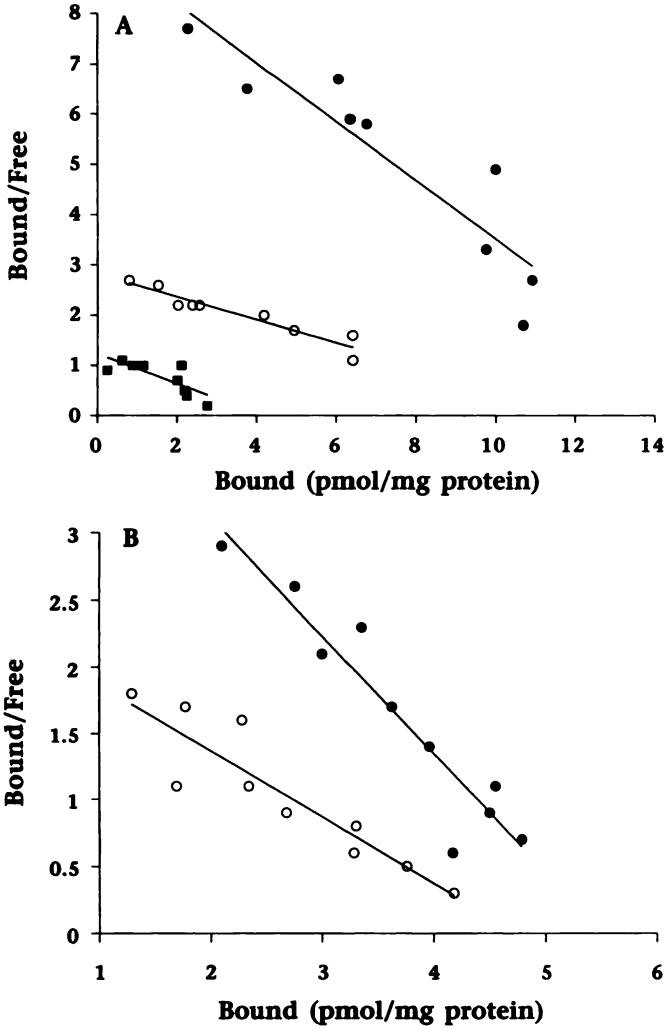

Fig. 3. Scatchard plots of [^{3}H]GBR 12935 binding to the DAUC in rat CPu. Data shown are values from one experiment. Values from several experiments are summarized in Table 1. A, Scatchard plots in MOPS buffer. In the absence of exogenous cation (○), the CPu had a K_d of 4.3 ± 0.9 nM (mean ± standard error) and a B_{max} of 11.6 ± 1.0 pmol/mg of protein (data shown represent values from a single experiment). Cd^{2+} (100 μM) (■) resulted in a significant ($p = 0.001$, Bonferroni correction for eight *post hoc* comparisons using $p < \alpha/n$, $\alpha = 0.05$, $n = 8$, or $p < 0.006$) decline in the B_{max} (4.8 ± 0.5 pmol/mg of protein) with no change in the K_d (4.0 ± 0.3 nM) ($p = 0.73$). Zn (50 μM) (●) resulted in a significant decrease ($p = 0.002$) in the K_d (1.5 ± 0.3 nM) and no change in the B_{max} (13.6 ± 1.4 pmol/mg of protein) ($p = 0.20$). Both Ni^{2+} (K_d = 1.9 ± 0.1 nM and B_{max} = 13.4 ± 0.5 pmol/mg of protein) and Co^{2+} (K_d = 1.9 ± 0.4 nM and B_{max} = 11.0 ± 1.3 pmol/mg of protein) produced significant decreases ($p = 0.003$ for Ni^{2+} and $p = 0.005$ for Co^{2+}) in the K_d but no change in the B_{max} ($p = 0.25$ for Ni^{2+} and $p = 0.71$ for Co^{2+}). B, Scatchard plots in the phosphate buffer. In the absence of exogenous cation (○), the CPu had a K_d of 2.1 ± 0.2 nM (mean ± standard error) and a B_{max} of 7.0 ± 1.1 pmol/mg of protein (data shown represent values from a single experiment). Zinc (50 μM) (●) resulted in a significant decrease (p = 0.01) in the K_d (1.0 ± 0.1 nM) without significantly changing the B_{max} (6.3 ± 0.2 pmol/mg of protein).

absence of zinc to 890 ± 150 nM in the presence of zinc (50 μM). For WIN 35,428, the K_H decreased significantly from 216 ± 25 nM in the absence of zinc to 52 ± 4 nM in the presence of zinc (50 μM). K_L did not change significantly in the presence of zinc (50 μM) for either cocaine or WIN 35,428.

NEM studies. NEM (5–100 mM) produced dose-dependent decline in the density of the DAUC (Fig. 6). This effect could

TABLE 1

Scatchard values in the absence and presence of different divalent cations

Saturation curves were generated using [^{3}H]GBR 12935 (0.1–10 nM) and analyzed as described in Materials and Methods. The sulfhydryl-reactive cation Cd^{2+} resulted in a significant decrease in the B_{max}, without changing the K_d. The three transition metal cations, Zn^{2+}, Ni^{2+}, and Co^{2+} (at the concentrations listed), each significantly decreased the K_d without changing the B_{max}. Values are the mean ± standard error from four independent experiments.

Ion	Concentration	K_d	B_{max}
	μM	nM	pmol/mg of protein
None	0	4.3 ± 0.9	11.6 ± 1.0
Cd^{2+}	100	4.0 ± 0.3	4.8 ± 0.5[a]
Zn^{2+}	50	1.5 ± 0.3[a]	13.6 ± 1.4
Ni^{2+}	500	1.9 ± 0.1[a]	13.4 ± 0.5
Co^{2+}	1000	1.9 ± 0.4[a]	11.0 ± 1.3

[a] Significantly different from control after Bonferroni correction of eight *post hoc* tests ($p < \alpha/n$, $\alpha = 0.05$, $n = 8$, or $p < 0.006$).

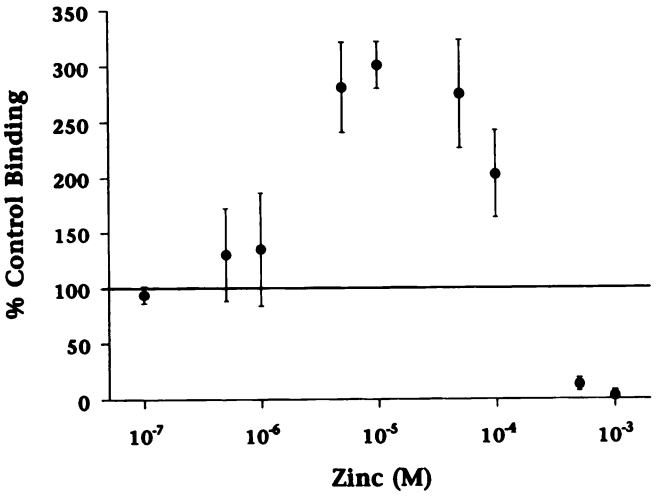

Fig. 4. Effect of different concentrations of zinc on binding of [^{3}H]GBR 12935 to the DAUC. The concentrations of zinc that increased binding ranged from 500 nM to 100 μM with albumin present in the incubation buffer. The lowest concentration that increased binding (500 nM) may represent one of the highest affinity neurotransmitter sites for zinc in a synapse. High concentrations of zinc (>100 μM) may reduce binding by oxidation of sulfhydryl groups.

be completely blocked by incubation with DTT (10 mM), mazindol (25 μM), or cocaine (100 μM).

DA uptake studies. DA uptake was dependent on tissue concentration, DA concentration, and time (data not shown). Uptake in the absence of blockers and ions averaged 1.72 ± 0.11 pmol/mg of protein/min (mean ± standard error from eight separate experiments). Zinc inhibited DA uptake at concentrations from 0.5 μM to 100 μM (Fig. 7). Cocaine inhibited DA uptake in a dose-dependent manner (Fig. 8). Cocaine inhibition of DA uptake was potentiated by the presence of zinc (Fig. 8). Cocaine (1 μM) resulted in 29% inhibition of uptake in the absence of zinc and 49% inhibition in the presence of zinc (0.5 μM). The sum of the effects of cocaine (29%) and zinc (3%) at those concentrations would be 32%. Thus, zinc enhanced inhibition of uptake by cocaine by 58% at these concentrations. The effect of zinc on cocaine inhibition of DA uptake occurred at different concentrations of cocaine and zinc. Cocaine at a low concentration (0.1 μM) resulted in no inhibition of DA uptake. However, in the presence of zinc (1 μM) cocaine (0.1 μM) produced 25% inhibition, which exceeded the effect of zinc alone (14%) by 79% (Fig. 8).

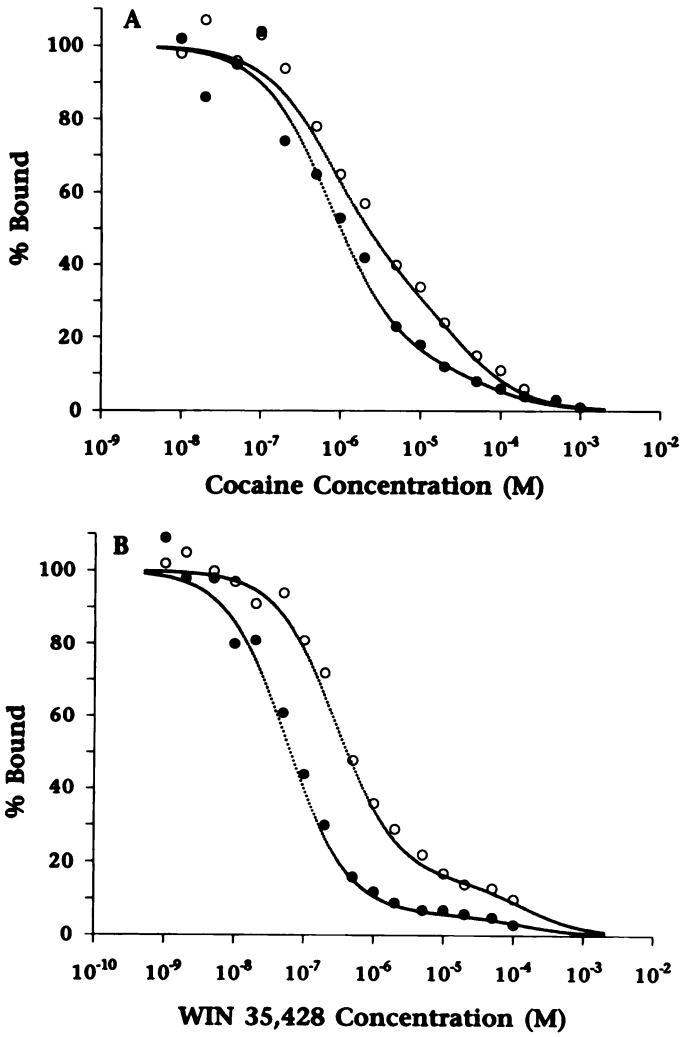

Fig. 5. Competition curves for cocaine and WIN 35,428 in rat CPu. Data points shown are from one experiment. Values from several experiments are summarized in Table 2. Curves are computer-generated best-fit lines derived from values obtained from the computer modeling of the data. A, Competition curves for cocaine (0.01–1000 μM) in the absence (○) or presence (●) of zinc (50 μM). Zinc increased the proportion of R_H from 73 ± 4 to 90 ± 2% and decreased the K_H from 2250 ± 880 nM to 890 ± 150 nM ($p = 0.02$; this value is very near the significance level of 0.017 using the conservative Bonferroni correction). The K_L was not significantly changed ($p = 0.54$) by the presence of zinc (70,400 ± 19,500 nM in the absence of zinc and 90,300 ± 17,900 nM in the presence of zinc). B, Competition curves for WIN 35,428 (0.001–100 μM) in the absence (○) or presence (●) of zinc (50 μM). Zinc significantly increased the proportion of R_H from 84 ± 1 to 93 ± 1% ($p = 0.0004$) and significantly decreased the K_H from 216 ± 25 nM to 52 ± 4 nM ($p = 0.003$). The K_L was not significantly increased by the presence of zinc from 67,600 ± 29,300 nM to 134,000 ± 123,000 nM ($p = 0.36$, due to high variance in the measure of K_L because of the low proportion of binding to that site, 7%, and the limited number of different concentrations used).

Discussion

Zinc enhancement of binding to the DAUC. This report demonstrates several novel findings related to binding sites on the DAUC. Three transition metal cations, Co^{2+}, Ni^{2+}, and Zn^{2+}, interact with the DAUC to increase its affinity for uptake blockers. These three cations increased the affinity of the DAUC without altering the B_{max}. At very high concentrations these cations may have other actions on the DAUC.

Zinc is present in the rat CPu in high concentrations (886 nmol/g of dry weight) (31). Zinc has access to synaptic clefts in CPu from two known sources. This cation may be coreleased with DA and ATP from DAergic terminals (32, 33) or may be released from synaptic terminals of corticofugal projections (34) as shown for the hippocampal mossy fiber pathways (5, 6). The estimated extracellular fluid concentration of zinc is about 10 nM (1, 2). Neuronal activation in the hippocampus leads to release of zinc at concentrations up to 300 μM (5–7). The concentration of zinc that increased the affinity of the DAUC and inhibited DA uptake in the present study (0.5–100 μM) falls well within this potential synaptic concentration range. These values suggest that, if zinc is actively released in the CPu, the achievable synaptic concentration likely falls within a range that may have a measurable effect on the DAUC. The concentration range of zinc that is active at the DAUC *in vivo* has yet to be determined.

The concentration range of zinc that is active at the DAUC is very similar to the concentration of zinc that is effective on amino acid neurotransmitter receptors, which ranges from 1 to 500 μM (9, 10, 56). Zinc has been shown to act on different amino acid neurotransmitter receptors including excitatory receptors (NMDA, quisqualate, and kainate receptors), inhibitory receptors (γ-aminobutyric acid receptors), and modulatory sites (glycine and phencyclidine binding sites on the NMDA receptor) (17, 35). Zinc enhances binding of a peptide neurotransmitter, human growth hormone, to the human prolactin receptor at low micromolar concentrations (36, 37). To this diverse list of receptor binding sites for zinc can be added a site on the DAUC. Zinc may function as a widespread modulator of neurotransmitter activity.

Zinc is likely to be the endogenous cation responsible for this effect in the CPu. Of the three transition metal cations examined, zinc had the highest apparent affinity for the uptake complex and produced the largest increase in [^{3}H]GBR 12935 binding (315% of control at 10 μM). Zinc also has known mechanisms for its storage and release within the CNS. Other endogenous cations (such as Fe^{2+} or Fe^{3+}) or exogenous cations (Pb^{2+}) may be able to interfere directly with the actions of Zn.

Zinc effects on cocaine affinity states. Perhaps the most interesting finding of this report relates to the interconversion of cocaine affinity states on the DAUC by zinc. Although the change in the proportion of R_H in rat CPu produced by zinc was small (9–17% increase in R_H), the effect was nonetheless consistent for two drugs and resulted in greater DA uptake inhibition by cocaine *in vitro*. Only one concentration of zinc was tested for alteration in cocaine affinity states. Thus, the maximal change in the proportion of the two affinity states caused by zinc may differ for other zinc concentrations. The concentration of zinc (50 μM) used for the competition studies was not the concentration producing the maximal alteration in binding (10 μM), and this concentration (50 μM) may have had some sulfhydryl-oxidizing effect. No attempt was made to remove or reduce endogenous zinc or other soluble substances that may modulate binding. However, the presence of two 10-μm-thick brain sections in 10 ml of buffer results in significant dilution of all endogenous soluble substances to very low concentrations. Other factors may interact with zinc on the DAUC to regulate affinity states as well.

Although the presence of two affinity states for cocaine has been known for several years (15–18, 20), the relationship between these states had not been clarified. The affinity states

TABLE 2

Competition values for the DAUC in the absence and presence of zinc

Competition curves were generated using [^{3}H]GBR 12935 (0.25 nM) and analyzed as described in Materials and Methods. Both cocaine and WIN 35,428 were best modeled as having two affinity states. Zinc (50 μM) had two effects on the binding characteristics of the DAUC for cocaine and WIN 35,428. First, zinc increased the proportion of R_H for each drug by 9–17% (decreasing the proportion of R_L by a comparable amount). Second, zinc decreased the K_H of the DAUC for cocaine and WIN 35,428. Values are the mean ± standard error for the number of experiments listed (*n*).

Competitor	*n*	n_H	K_H	R_H	K_L	R_L
			nM	%	nM	%
No zinc						
Cocaine	6	0.67 ± 0.02	2,250 ± 880	73 ± 4	70,400 ± 19,500	27 ± 4
WIN 35,428	3	0.56 ± 0.03	216 ± 25	84 ± 1	67,600 ± 29,300	16 ± 1
Zinc (50 μM)						
Cocaine	3	0.69 ± 0.06	890 ± 150[a]	90 ± 2[b]	90,300 ± 17,900	10 ± 2
WIN 35,428	3	0.70 ± 0.09	52 ± 4[b]	93 ± 1[b]	134,000 ± 123,000	7 ± 1

[a] Nearly significant ($p = 0.02$), despite the most conservative correction (Bonferroni) for multiple *post hoc* tests.
[b] Significantly different, compared with values in the absence of zinc, using a Bonferroni correction for three *post hoc* tests ($p < \alpha/n$, $\alpha = 0.05$, $n = 3$, or $p < 0.017$).

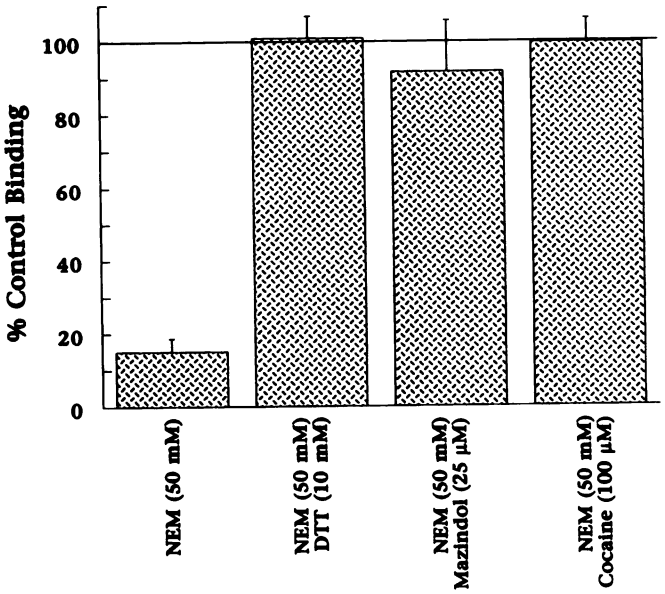

Fig. 6. Effect of NEM on binding to the DAUC. NEM (50 mM) reduced binding to the DAUC by 85%. This reduction was dose dependent, with concentrations as low as 5 mM resulting in loss of binding (data not shown). The sulfhydryl-oxidizing effect on the DAUC could be reversed by incubation with DTT, cocaine, or mazindol. Incubation with DTT alone resulted in no change in binding (data not shown). Data are means and standard errors from three separate experiments.

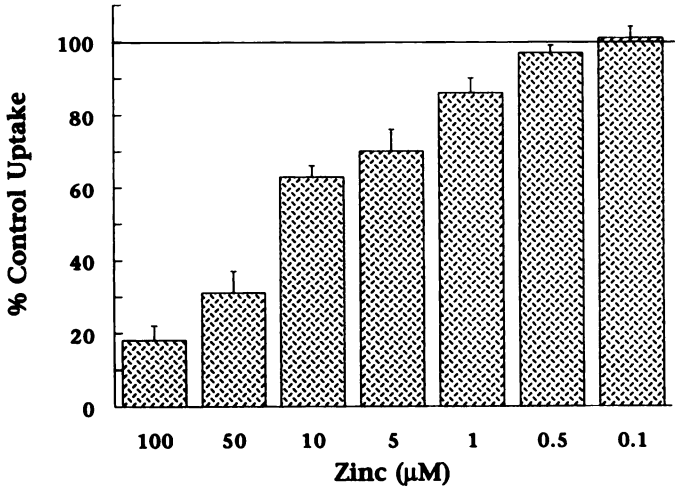

Fig. 7. Zinc inhibition of DA uptake. Zinc inhibited DA uptake in a dose-dependent manner at concentrations from 0.5 to 100 μM. Inhibition ranged from 3% (0.5 μM zinc) to 72% (100 μM zinc). Data are the means and standard errors from three to six separate experiments.

reported here differ in several ways from those reported by others. First, the proportions of R_H for cocaine and WIN 35,428 (73–84% in the absence of zinc and 90–93% in the presence of zinc) described here are higher than those reported by others (4–13%) using different ligands ([^{3}H]cocaine, [^{3}H]WIN 35,065-2, and [^{3}H]WIN 35,428) and homogenate preparations (15, 16, 20). This may be due to technical reasons, in that all data reported from this study relied on autoradiography of tissue sections, which may preserve integral membrane components or structure. This study also used competition analysis of an unlabeled drug rather than direct saturation analysis. Finally, this study used [^{3}H]GBR 12935, which does not discern the two affinity states by itself but which can be used in cocaine and cocaine congener competition analysis. This difference in proportion of affinity states is not unlike the greater proportion of R_H for DA D-2 receptors found using autoradiography compared with homogenate preparations (38).

Another difference between this study and others (15, 16, 20) is in the affinity constants for cocaine and WIN 35,428. The K_H values for cocaine and WIN 35,428 (2250 nM and 216 nM in the absence of zinc and 890 nM and 52 nM in the presence of zinc, respectively) found in this study are higher than those reported by others (19 nM for cocaine and 4.7 nM for WIN 35,428) (15, 16). The K_L values for cocaine and WIN 35,428 (70.4–90.3 μM and 67.6–134 μM, respectively) are also higher than those reported (1.1 μM for cocaine and 60 nM for WIN 35,428) by others (15, 16). This may reflect the long incubation time (30 hr) used in this assay, which may have resulted in some degradation of drug over time. A second factor may relate to the presence of albumin (0.01%) in the buffer, which may minimally lower free concentration of the compounds tested. Alternatively, homogenate preparations may result in release of endogenous zinc, which may increase the affinity of the DAUC for the different ligands. An additional possibility is the presence of other soluble factors that are present in homogenate preparations at a sufficient concentration to alter binding properties of the DAUC but are present at too low a concentration in this autoradiographic assay.

It is unlikely that the two affinity states recognized in this assay represent binding to sites other than on the DAUC, for several reasons. First, displacement of binding using a variety of compounds active on the DAUC occurred with a rank order

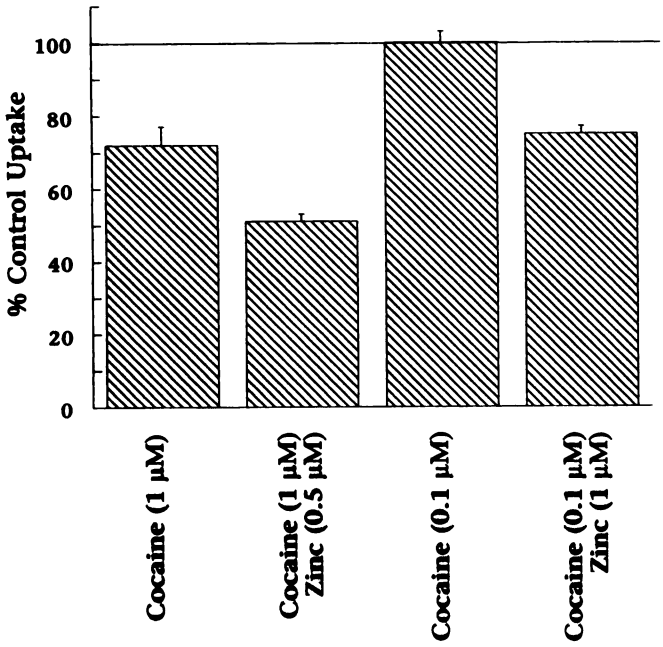

Fig. 8. Zinc enhancement of cocaine inhibition of DA uptake. Cocaine (1 μM) resulted in a 28% inhibition of DA uptake. In the presence of zinc (0.5 μM) cocaine (1 μM) produced 49% inhibition of uptake, whereas zinc (0.5 μM) alone resulted in 3% inhibition. The sum of their independent inhibitions would be 31%. Zinc (0.5 μM), therefore, resulted in a 58% increase in inhibition by cocaine (1 μM). Augmentation of cocaine inhibition was seen at another concentration of cocaine and zinc. Zinc (1 μM) enhanced cocaine (0.1 μM) inhibition by 79%, from no inhibition in the absence of zinc to 25% inhibition in the presence of zinc. Zinc (1 μM) alone produced 14% inhibition of DA uptake. Values are the means and standard errors from five separate experiments.

of potency consistent with that for the DAUC (18). Second, although [^{3}H]GBR 12935 can bind to a variety of sites including the DAUC, a piperazine acceptor site (39, 40), and serotonin and noradrenergic uptake complexes (41, 42), the conditions used in this report were designed for selective binding to the DAUC. This was achieved by using a concentration of [^{3}H] GBR 12935 (0.25 nM) that does not label the other uptake complexes and by including *trans*-flupentixol (0.75 μM), which blocks 97% of the piperazine acceptor sites (18), in the buffer. Third, the maximal degree of displacement (specific binding) occurred to the same level (near film background) with all compounds tested that are active at the DAUC, including those recognizing one or two sites. Fourth, mazindol (a potent DAUC inhibitor) was used in all experiments to define nonspecific binding. Fifth, the ability of zinc to alter both the proportion of the two affinity states and the high affinity constant suggests that these two states are linked together and likely represent similar related binding sites. A final consideration is that the DAUC and the cocaine recognition site may function as a three-site model, with only two states being recognized by different types of assays. Insufficient data points were available for the competition curves in this study to allow testing of a three-site model. Further work using homogenate and autoradiographic assays may clarify this issue. The cloning and *in vitro* characterization of expressed protein(s) for the DAUC may also clarify this issue (43, 44).

A zinc allosteric site is also present in human striatum (17). It is of interest that the proportion of high affinity sites for cocaine in the absence of zinc is greater in the rat CPu (73%) than in human putamen (54%). Also, zinc (50 μM) produced a greater increase in the proportion of cocaine R_H in human putamen (20% increase in R_H for WIN 35,428 and 21% increase for cocaine) than in rat CPu (9% increase in R_H for WIN 35,428 and 17% increase for cocaine).[1] These findings suggest that there may be sequence homologies and possibly differences between the human and rat genes for the DAUC.

Zinc effect on DA uptake. Zinc was able to inhibit DA uptake directly in the absence of other uptake blockers. The concentrations required for uptake inhibition (0.5–100 μM) are above basal levels (10 nM) and below those achieved by maximal neuronal stimulation (300 μM). Endogenous synaptic zinc may thus be considered a direct modulator of DA uptake. Whether the binding site for zinc is in the channel that transports DA or is distant, possibly on one of the cytoplasmic loops, is not currently known. This site, however, appears to induce a conformational change in the uptake complex that results in several effects. These include a direct reduction in DA uptake and an indirect reduction in DA uptake by enhancement of the binding of cocaine and other uptake blockers to the DAUC and shifting of the DAUC to a greater proportion of R_H. The binding of an endogenous uptake inhibitor in brain might also be affected by zinc.

Possible mechanism for zinc interaction with the DAUC. Interactions between corticostriatal glutamate and nigrostriatal DA systems have been reported and mechanisms postulated (45, 46). Glutamate promotes release of DA from DA terminals acting via NMDA or kainate receptors (47–49). Zinc is present in synaptic vesicles of excitatory synaptic boutons (8, 50) and may be released from corticostriatal excitatory amino acid-containing neurons (3, 6). Spontaneous release of zinc in the synaptic cleft may also occur (4, 51). Corticostriatal terminals form asymmetric synaptic contacts on distal dendritic spines of medium spiny neurons (52, 53). A portion of the DAergic nigrostriatal terminals form symmetric contacts on the same distal dendritic spines of medium spiny neurons receiving corticostriatal contacts (52, 54, 55). If zinc is able to diffuse from the corticostriatal terminal on a dendritic spine to the nigrostriatal terminal on the same spine, it might account for zinc modulation of the DAUC (volume transmission). Corticostriatal activity near a DA terminal might be synergistic by both directly releasing DA (glutamate acting on NMDA receptors) and by blocking the reuptake of DA (zinc acting on the DAUC). Although this report does not demonstrate activity of zinc on the DAUC *in vivo*, the *in vitro* data support these potential mechanisms for corticostriatal modulation of DA input to the CPu.

Potential behavioral actions of zinc on the DAUC. Some of the behavioral effects produced by cocaine, including tolerance and sensitization (22, 24, 25), may relate to cocaine affinity states. Zinc may play a role in modulating DA uptake blockade by cocaine *in vivo*. Zinc is capable of enhancing the binding of cocaine to the DAUC. This effect is significant in terms of both increasing the affinity of the complex for cocaine and inhibiting DA uptake. Concentrations of cocaine (0.1 μM) that are too low to block reuptake *in vitro* in the absence of zinc result in block in the presence of zinc. Elevated concentrations of synaptic zinc would increase both the proportion of high affinity sites for cocaine and the affinity of the DAUC for

[1] E. K. Richfield, unpublished observations.

cocaine. These two effects might dramatically increase the degree of DA uptake blockade by a given synaptic concentration of cocaine *in vivo*. Conversely, if the synaptic concentration of zinc is reduced by neural mechanisms, this would have the opposite effect of reducing the degree of uptake blockade by cocaine.

If corticostriatal zinc pathways play a role *in vivo* in the action of DA reuptake in the striatum, it suggests that higher cortical activity may be able to modulate synaptic DAergic activity. Corticostriatal activity may enhance DA activity in the striatum and might become altered under pathological conditions. Excess zinc activity would promote DAergic activity; zinc deficiency would decrease synaptic DA and allow greater uptake and perhaps neurotoxicity. Several avenues of potential interruption of zinc actions on the DAUC exist. Depletion of endogenous zinc may be possible. Drugs designed to alter or compete at the zinc domain on the DAUC would alter DA uptake. Drugs might be able to modify the interconversion of cocaine affinity states. The recent isolation of a gene coding for the DAUC provides for the primary structure of the complex, which will ultimately lead to identification of the zinc binding site. Drugs designed to modify the zinc-binding domain on the DAUC may prove therapeutic in a variety of disorders related to altered synaptic DA concentration. Further understanding of the actions of zinc *in vivo* will be required before rational drug design may be possible.

Effect of different buffers on binding to the DAUC. Different buffers affect the binding of [^{3}H]GBR 12935 to the DAUC. Changes in density at a single concentration of [^{3}H]GBR 12935 (0.25 nM) suggest that all buffers may influence binding to the DAUC. Alterations in binding may occur via a change in the B_{max}, K_d or both when two buffers are compared. The MOPS buffer increased both the K_d and B_{max} of the DAUC for [^{3}H]GBR 12935, compared with the phosphate buffer. These findings support the need for careful characterization of assay characteristics before other types of studies. Despite differences seen with the two buffers used, all changes seen with cations were always compared with control conditions in the absence of the added cation. In addition, the effect of zinc on binding of [^{3}H]GBR 12935 to the DAUC was similar for the two buffers examined.

Role of sulfhydryl groups on the DAUC. Sulfhydryl groups have been found to play a role in binding to the DAUC (57). This report supports that finding by demonstrating that the rank order of potency of a set of cations ($Hg^{2+} > Cu^{2+} > Cd^{2+}$) reactive at sulfhydryl groups is appropriate for that activity. The oxidation by metal cations can be reversed by reducing agents.[1] Reductive alkylation of sulfhydryl groups by NEM decreased binding of [^{3}H]GBR 12935 to the DAUC and was prevented by pretreatment with uptake blockers. Sequence data for the DAUC demonstrate cysteine residues on two cytoplasmic loops of the DAUC that may be involved in the binding of uptake blockers. It remains to be determined whether reduced sulfhydryl groups contribute to the affinity states for cocaine or are involved in the binding of zinc to the DAUC.

Conclusions. Zinc appears to play an important role regulating both drug binding and DA uptake by the DAUC. Zinc increased the affinity of the DAUC for uptake blockers and altered the proportion of high and low cocaine affinity states. Zinc directly inhibited DA uptake and potentiated cocaine inhibition of DA uptake at physiologically relevant synaptic concentrations.

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