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Binding Domains for Blockers and Substrates on the Cloned Human Dopamine Transporter Studied by Protection Against *N*-Ethylmaleimide-Induced Reduction of 2β-Carbomethoxy-3β-(4-fluorophenyl)[^{3}H]tropane ([^{3}H]WIN 35,428) Binding

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ABSTRACT. Binding sites for 2β-carbomethoxy-3β-(4-fluorophenyl)[^{3}H]tropane ([^{3}H]WIN 35,428) on the human dopamine transporter expressed in C6 glioma cells were alkylated with *N*-ethylmaleimide (NEM), and the protective potency of the blockers cocaine, *N*[1-(2-benzo[b]thiophenyl)cyclohexyl]piperidine (BTCP), and benztropine, and of the substrates dopamine, *d*-amphetamine, and norepinephrine was measured. In general, the protective potency was lower (at least 4–5 times) than the potency in inhibiting [^{3}H]WIN 35,428 binding with the compounds present under the same experimental conditions used for the NEM alkylation. However, the disparity was substantially greater for all substrates tested (23- to 44-fold) than for the blockers (4- to 11-fold), especially cocaine (5-fold) and BTCP (4-fold). Benztropine took an intermediate place (11-fold) between cocaine (5-fold) and BTCP (4-fold), on the one hand, and dopamine (23-fold), on the other hand. [^{3}H]WIN 35,428 binding was best described by a one-site model under the present conditions. The results are discussed in terms of models involving blocker-induced conformational changes and overlapping nonidentical binding domains for blockers and substrates. BIOCHEM PHARMACOL 52;9:1435–1446, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. WIN 35,428 binding; dopamine binding; cocaine binding; protein modification; *N*-ethylmaleimide; human dopamine transporter

Inactivation of extracellular dopamine occurs by uptake of the neurotransmitter into nerve terminals by the DAT,† a member of the Na^+- and Cl^--dependent neurotransmitter transporters which include the three subfamilies of monoamine, γ-aminobutyric acid, and amino acid transporters [1–3]. The DAT is a target for drugs such as cocaine or *d*-amphetamine and the neurotoxin *N*-methyl-4-phenylpyridinium (for references, see Ross [4] and Giros and Caron [2]). In interacting with the DAT, compounds can be classified as substrates, gaining access to the opposite side of the plasma membrane by translocation, or blockers, causing a conformational state incapable of translocation (for references see Zimanyi *et al.* [5]). The binding domains on the transporter involved in the interaction with substrates may not be identical to those for blockers. This would allow for a putative cocaine antagonist that does not by itself block dopamine uptake, but does interfere with the action of cocaine [6–8]. It would also be consonant with the concept advanced by Rothman [9] of partial agonism as applied to uptake carriers. Accordingly, there are substances (termed "type 2") that only partially inhibit dopamine uptake and can, in fact, reduce the effectiveness of a blocker such as cocaine (a "type 1" compound) [9, 10].

The observation that dopamine is substantially weaker in inhibiting binding of radiolabeled blockers to the dopamine uptake complex than in inhibiting its own translocation in uptake assays [11–13] can be interpreted as supportive of the existence of separate blocker and substrate sites. Alter-

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† *Abbreviations:* DAT, dopamine transporter; hDAT, human dopamine transporter; BTCP, *N*[1-(2-benzo[b]thiophenyl)cyclohexyl]piperidine; GBR 12909, 1-(2-(di(4-fluorophenyl)-methoxy)-ethyl)-4-(3-phenylpropyl)piperazine; GBR 12935, 1-(2-(diphenylmethoxy)ethyl)-4-(3-phenylpropyl)piperazine; GBR 12783, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenyl-2-propenyl)piperazine; RTI-55, 3β-[4′-iodophenyl]tropan-2β-carboxylic acid methyl ester; WIN 35,428, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane; NEM, *N*-ethylmaleimide; PC_{50}, concentration needed to provide 50% protection against NEM attack; K_m, substrate concentration that produces half-maximal transport velocity; K_d, equilibrium dissociation constant; and B_{max}, density of binding sites.

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natively, it can be explained by a conformational model with a rate-limiting reorientation step that causes the K_m for dopamine uptake to be lower than the K_d for substrate binding [5] analogous to models developed for the norepinephrine [14] and serotonin [15, 16] transporter. In favor of the latter model, we observed mutually exclusive binding of the phenyltropane analog of cocaine, WIN 35,428, and dopamine in equilibrium binding experiments [17]. In addition, a number of studies on dopamine translocation into rat striatal nerve terminals have described a competitive inhibition by various uptake blockers including cocaine [18–20], and recent *in vivo* [21] and *in vitro* [22] voltammetric measurements in rat striatum on a sec-time scale were reported to confirm the competitive nature of inhibition for nomifensine, cocaine, and other compounds. However, rotating disk voltammetry measurements in rat striatal suspensions on a sec-time scale [23] and model fitting of [^{3}H]dopamine uptake into striatal synaptosomes [24] suggested an uncompetitive interaction of cocaine with the DAT involving Na^+ binding sites. In addition, site-directed mutagenesis techniques have been used to demonstrate the importance of Asp^{79} in transmembrane domain 1 for both dopamine and WIN 35,428 interaction [25] and of Tyr^{250} in domain 4 in interacting with WIN 35,428 [26], whereas Ser^{356} and Ser^{359} in domain 7 have been implicated in dopamine interactions [25]. The observation that mutation of Ser^{507}, Ser^{518}, and Tyr^{513} in domain 11 to Ala preferentially increased uptake of the substrate *N*-methyl-4-phenylpyridinium [27] is consonant with the results from experiments with chimeric dopamine-norepinephrine transporters showing that inhibition of dopamine uptake by substrates, but not blockers, involves the carboxyl-terminal region encompassing transmembrane domain 9 through the carboxyl end [28]. If uptake blockers and substrates recognize nonidentical but overlapping binding domains on the DAT, it may be difficult to address the nonidentical domains in mutual inhibition studies of the type we attempted previously [17, 29]. In a different approach, Wall *et al.* [30] observed a difference between 3β-[4′-[^{125}I]iodophenyl]tropan-2β-carboxylic acid methyl ester ([^{125}I]RTI-55) (resembling WIN 35,428 with iodine instead of fluor in the 4-phenyl position) and dopamine in their dependency upon Cl^- and H^+ in competition experiments with rat striatal membranes, suggesting the involvement of nonidentical binding domains; however, the ion dependencies themselves may need to be reinterpreted while taking into account the known inhibitory effect of Li^+, used as the substitution ion for Na^+, on cocaine and cocaine analog binding [31, 32].

Johnson *et al.* [33] concluded that a significant portion of the cocaine binding domain is distinct from that of the substrates dopamine or *d*-amphetamine based on a differential protection offered by these compounds against alkylation by NEM of [^{3}H]mazindol binding sites on rat striatal membranes. However, dopamine, *d*-amphetamine, or *m*-tyramine, another substrate, were not relatively weaker in protecting 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenyl-2-[^{3}H]propenyl)piperazine ([^{3}H]GBR 12783) binding sites against NEM-induced alkylation than cocaine or other blockers when taking into account the affinity of the compounds for the DAT measured under the same conditions in the same laboratory as reported by Heron *et al.* [34]. Although the different results from the two studies could be related to the use of different radioligands perhaps labeling nonidentical binding domains, it should be kept in mind that the protective potencies in the study by Johnson *et al.* [33] were not obtained under the same experimental conditions (preparation, assay buffer, pH) as the inhibitory potencies used for comparison [5, 35], and the importance of these conditions has become increasingly clear both for blockers [5, 32, 36–42] and substrates [30]. In the present study, the NEM alkylation approach was applied to the hDAT cloned by Janowsky and co-workers [43, 44]. [^{3}H]WIN 35,428 was used as the radioligand, and protective as well as inhibitory potencies of compounds were determined under the same experimental conditions in order to facilitate comparison.

MATERIALS AND METHODS

Materials

[^{3}H]WIN 35,428 (84.5 Ci/mmol) was obtained from Dupont-New England Nuclear (Boston, MA). Cocaine hydrochloride was from the Mallinckrodt Chemical Corp. (St. Louis, MO). GBR 12909 and WIN 35,428 were from Research Biochemicals Inc. (Natick, MA). GBR 12935 and BTCP were synthesized by Drs. Brian de Costa, Kenner C. Rice, and A. E. Jacobson (NIDDK, NIH, Bethesda, MD). *d*-Amphetamine from Smith, Kline & French (Philadelphia, PA) was in the sulfate form; concentrations are expressed as moles of the sulfate salt which contains 2 moles of *d*-amphetamine. All other chemicals were from Sigma (St. Louis, MO) or Fisher (Springfield, NJ).

C6 Glioma Cells Stably Expressing hDAT

hDAT cDNA was cloned by screening a human substantia nigra cDNA library with a polymerase chain reaction-amplified probe based on the rat DAT cDNA sequence, and stably transfected into rat C6 glioma cells, in the laboratory of Dr. Aaron Janowsky (Oregon Health Sciences University, Portland, OR) as described previously [43, 44]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum and 5% bovine calf serum; in the case of the transfected cells, the medium also contained 2 μg/mL puromycin. Frozen aliquots were prepared in a medium containing 45% DMEM, 5% DMSO and 50% fetal bovine serum at a density of 10^7 cells/mL (C6-hDAT) and 4.5×10^6 cells/mL (untransfected). For each experiment, one freezing vial was rapidly thawed and seeded into a 75-cm^2 flask at a density of approximately 100,000 cells/cm^2. When the flask reached confluency (after ≈4 days at a density of ≈600,000

cells/cm^2), the cells were lysed by trypsinization and seeded into four 75-cm^2 flasks. Approximately 4 days later, when these four flasks reached confluency (600,000 cells/cm^2), the cells were used for an experiment.

A growth curve was established by seeding different 25-cm^2 flasks and counting cells at varying time intervals. We also tested for [^{3}H]WIN 35,428 binding during this time.

Preparation of Membranes for Binding Experiments

The medium was removed from the cells, and the cells were washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and lysed with 2 mM HEPES, 1 mM EDTA, pH 7.6, at room temperature (3 mL/flask, on ice for 10 min). The lysate was transferred to a centrifuge tube, and the flask was washed out with an additional 2 mL of the lysis buffer. After centrifugation of the lysate at 31,000 *g* for 20 min, the pellet was homogenized with a Brinkmann polytron (setting 6, 15 sec) in 3.5 to 4 mL of the same buffer used for the binding assay (see below). Approximately one flask-equivalent of cells was used for each inhibition analysis involving various concentrations of a given compound as detailed below; two flask-equivalents were needed per compound for the protection experiments.

Saturation Analysis of [^{3}H]WIN 35,428 *Binding and Inhibition by d-Amphetamine*

Membranes were processed as in the protection experiments described in the next section (preincubation and treatment in the presence or absence of 6 mM NEM). Subsequently, binding assays were carried out in triplicate in a total volume of 0.2 mL in 1-mL ministrip tubes (Skatron, Sterling, VA). The final concentrations were 25 mM sodium phosphate [from 12.5 mM secondary phosphate buffer adjusted to a pH of 7.4 at room temperature by 0.85% (v/v) H_3PO_4], 102 mM NaCl, 2.5 nM [^{3}H]WIN 35,428, various concentrations of WIN 35,428 (0, 1.5, 5, 15, 50, and 150 nM), and 0.1 to 0.2 mg of C6-hDAT (or parental untransfected C6) membrane protein (50 μL of suspension). The assay mixture was incubated in a shaker at 0–4° for 2 hr. The reaction was terminated by the addition of 0.8 mL of ice-cold wash buffer (30 mM sodium phosphate buffer, pH 7.4, at room temperature) and filtration over Skatron receptor binding filtermats (glass fiber filter, 1 μm retention, No. 11734, equivalent to Whatman GF/B) with a miniharvesting apparatus (type 11021, Skatron). Filters were pretreated with 0.05% (w/v) poly-L-lysine. After the first filtration, filters were washed three times with 1 mL of ice-cold wash buffer, and assayed for radioactivity by liquid scintillation counting [Beckman model LS 6000IC spectrometer at 45% efficiency for filters in Cytoscint (ICN, Costa Mesa, CA) solution]. Nonspecific binding was defined with 30 μM cocaine, and amounted to 2% of the total value determined in the absence of inhibitor. Protein content was estimated by the Folin phenol reagent method as described previously [5].

In parallel to the above [^{3}H]WIN 35,428 saturation analysis, inhibition curves were run for *d*-amphetamine with six concentrations bracketing the IC_{50} value at a radioligand concentration of 2.5 nM.

Protection of Binding of [^{3}H]WIN 35,428 *against NEM Attack*

The final concentration of buffer components in these experiments was 25 mM sodium phosphate (resulting from mixing primary and half-strength secondary sodium phosphate buffer to pH 7.4 at room temperature) and 102 mM NaCl. One milliliter of membrane suspension (see "preparation of membranes" above) was pipetted into a centrifuge tube, 53 μL of drug stock (20-fold concentrated compared with desired final concentration) was added, and the mixture was incubated for 50 min at 0–4°. In experiments with substrates, the medium also contained 9 μM nialamide. Subsequently, 50 μL NEM (22-fold more concentrated than the desired concentration which was routinely 6 mM) was added, and the incubation was continued for 30 min at 0–4°. The incubation was terminated by addition of 4 mL of ice-cold buffer and centrifugation at 50,000 *g* for 20 min. The pellet was homogenized with the polytron (setting 6, 15 sec) in 3 mL of buffer, and centrifuged again. This washing step was repeated once more, and the final pellet was homogenized with the polytron in 0.5 mL of buffer. Aliquots of 50 μL of this suspension (20–40 μg of protein) were pipetted into 1-mL ministrip tubes (Skatron) containing buffer and [^{3}H]WIN 35,428. In a final volume of 0.2 mL and at a radioligand concentration of 4.3 nM, assay mixtures were incubated for 50 min at 0–4° in a shaker. For each membrane suspension, one triplicate was for the estimate of total binding and another triplicate contained 30 μM cocaine for the assessment of nonspecific binding. The protein content of each suspension was determined as described above, and all binding data were calculated as disintegrations per minute specifically bound per microgram of protein. Within each experiment, there were three incubations without NEM or compound (non-alkylated unprotected case), one incubation with NEM but without compound (alkylated unprotected case), and a number of incubations with NEM and various concentrations of compound (alkylated protected case). Non-alkylated controls for wash-out of compound were included periodically.

Inhibition of Binding of [^{3}H]WIN 35,428 *by Compounds*

With the same buffer as used for the NEM protection experiments, compounds were assayed for their potency in inhibiting [^{3}H]WIN 35,428 binding (0.45 nM) for 50 min at 0–4° in a final volume of 0.6 mL. Five to six concentrations of compound bracketing its IC_{50} value were tested, and nonspecific binding was defined with 30 μM cocaine. Substrates were tested in the presence of 9 μM nialamide as in the protection experiments.

Data Analysis

IC_{50} values and pseudo-Hill numbers were computed with the equation of the ALLFIT program of De Lean *et al.* [45] entered into the Microsoft ORIGIN curve-fitting and plotting software. This nonlinear regression program was run with nonspecific binding entered as a constant; the zero drug concentration was entered as a data point, and total binding was allowed to be fitted by the program. Equilibrium binding data were analyzed with the nonlinear computer fitting program LIGAND [46]. Data files in which nonspecific uptake (binding) (N_1) had not been subtracted were used; the results shown were obtained by entering N_1 as a constant in the fitting procedures and were usually close to estimates obtained by having N_1 float. In the protection experiments, all results were calculated using specific disintegrations per minute bound per microgram of protein. The three "Total" values (non-alkylated unprotected case) were averaged, and the "NEM" value (alkylated unprotected case) was noted. To calculate percent protection for each drug concentration (alkylated protected case, "Sample"), the following equation was used: (Sample − NEM)/(Total − NEM). The PC_{50} values were computed with ORIGIN in the same way as IC_{50} values as described above; the zero compound condition was entered as a data point, and the "Total" value was again allowed to be fitted by the program. Statistical analysis consisted of one-way ANOVA followed by the Least Significance Difference Multiple Range test for multiple comparisons. Where necessary, data were subjected to logarithmic transformation for homogeneity of variance prior to statistical analysis. Hill numbers were compared to unity using the single-sample *t*-test. The accepted level of significance was 0.05.

RESULTS

Cell Growth and [^{3}H]WIN 35,428 Binding

The growth curve of the C6 glioma cells had an approximate doubling time of 28 h. Transporter density measured by [^{3}H]WIN 35,428 binding indicated no significant changes as a function of culture duration (data not shown). Wild-type C6 cells grown as controls under the same conditions displayed very low levels of binding [3.7 ± 0.8 fmol of total binding/mg protein and 3.4 ± 0.9 fmol of nonspecific binding/mg protein (mean ± SEM for 3 independent preparations of cell membranes)] when compared with the binding levels of C6 hDAT cells tested in the same experiments [265 ± 60 fmol of total binding/mg protein and 5.3 ± 0.2 fmol of nonspecific binding/mg protein (for 3 preparations)].

Reduction of [^{3}H]WIN 35,428 Binding by NEM

The binding of [^{3}H]WIN 35,428 to C6-hDAT membranes was reduced by NEM in a concentration-dependent fashion after exposure to NEM and removal by repeated washing (Fig. 1). In the following experiments, a concentration of 6 mM NEM was used, resulting in a 76 ± 2% (mean ± SEM for 18 experiments) destruction of binding.

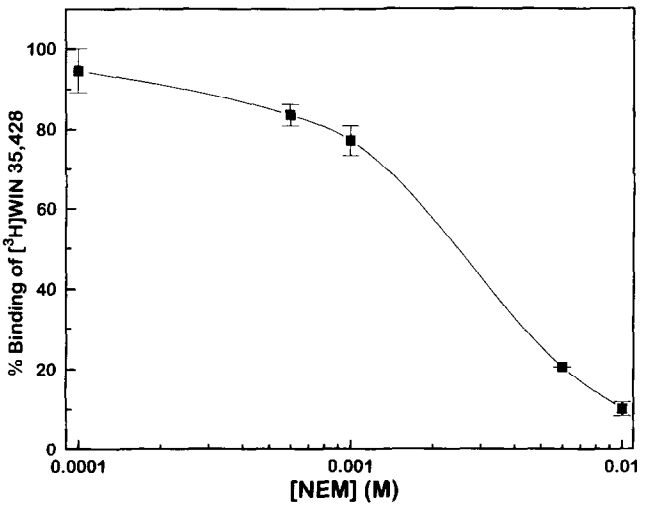

FIG. 1. Alkylation of [^{3}H]WIN 35,428 binding sites by various concentrations of NEM. C6-hDAT membranes were exposed to various concentrations of NEM in sodium phosphate buffer containing 102 mM NaCl for 30 min at 0–4°. After removal of NEM by repeated washing/centrifugation steps, [^{3}H]WIN 35,428 binding (4 nM) was measured. Binding in NEM-pretreated samples was expressed as a percentage of that measured in control samples not exposed to NEM but otherwise treated the same way. Values are means ± SEM of 3 independent experiments carried out in triplicate. The average control specific binding was 532 fmol/mg protein.

Saturation analysis of [^{3}H]WIN 35,428 binding was performed on both control- and NEM-pretreated cell membranes under conditions as used for the protection experiments presented below. The binding was best described by a one-site model as analyzed by the LIGAND program (Fig. 2A). All experiments together indicated no change in the K_d value (10–13 nM) and a reduction in the B_{max} value (from 2.16 to 0.94 pmol/mg protein) (Table 1).

In the same experiments, the potency of *d*-amphetamine was assessed. Pretreatment with NEM did not shift the inhibition curve of *d*-amphetamine (Fig. 2B) with all results taken together indicating a K_i of 3.0 to 3.2 μM (Table 1).

Protection against NEM

The NEM-induced decrease in binding was counteracted by various blockers and substrates chosen for the current study (Fig. 3). All compounds offered full to nearly full protection (80–100%) in the concentration range studied. No evidence was found for incomplete wash-out for these compounds which would have resulted also in incomplete protections at high concentrations.

Comparison between Protecting and Inhibitory Potency

Because the following protection/inhibition experiments were carried out with 50-min binding assays, we compared

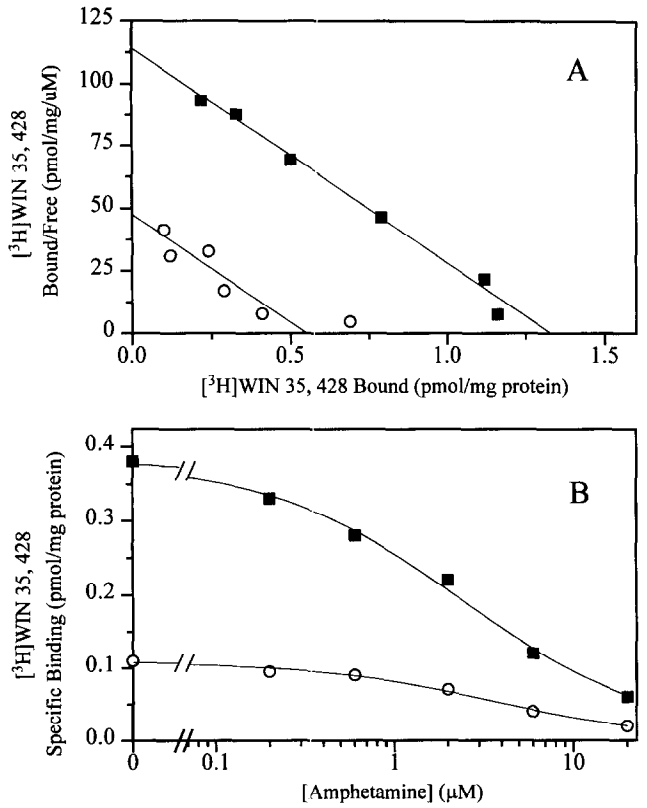

FIG. 2. Saturation analysis of [^{3}H]WIN 35,428 binding (A) and inhibition curves for *d*-amphetamine (B) after pretreatment in the presence (○—○) or absence (■—■) of 6 mM NEM. (A) The radioligand was present at 2.6 nM, and increasing concentrations of unlabeled WIN 35,428 were added up to 150 nM; nonspecific binding was defined with 30 μM cocaine. The straight line represents the best fit chosen by the LIGAND program. (B) The radioligand was present at 2.6 nM with increasing concentrations of *d*-amphetamine. Both panels show a typical experiment that was assayed in triplicate. The experiment was carried out three times with independent C6-hDAT membrane preparations (for averages see Table 1).

this assay time to the 120-min assay used in our previous studies [17, 29, 32, 37, 38] as well as for above saturation curves in this study; a parallel comparison with the same cell preparation showed virtually equal binding (data not shown), indicating that [^{3}H]WIN 35,428 binding reached equilibration within 50 min under the present conditions.

The inhibition curves for the compounds studied were monophasic (Fig. 3) with Hill numbers close to unity (Table 2). Clearly, for all compounds a higher concentration was needed to provide 50% protection against NEM attack than to cause 50% inhibition in equilibrium binding experiments (Fig. 3, Table 2). However, the disparity was greater for the substrates *d*-amphetamine, dopamine, and norepinephrine than for the blockers cocaine, BTCP, and benztropine. Thus, the ratio of PC_{50} over IC_{50} was significantly greater for any of the substrates (23–44) than blockers (4–11) (Fig. 4). Benztropine took an intermediate place being different (ratio of 11) from both BTCP (4) or cocaine (5) and from dopamine (23) or *d*-amphetamine (35) or norepinephrine (44) (Fig. 4).

DISCUSSION

Binding of [^{3}H]WIN 35,428 to C6-hDAT Cell Membranes; One Versus Two Sites

The generation time of approximately 1.2 days observed for C6-hDAT cells under the current conditions is in agreement with the generation time of 1.5 days reported for the original C-6 cells derived from rat brain glial tumors producing soluble S-100 protein [48]. The binding of [^{3}H]WIN 35,428 per unit of cell membrane protein was similar at a time before confluency was reached (approximately 5.5×10^5 cells/cm^2) and at confluency (approximately 7.8×10^5 cells/cm^2) (with the cells being fed daily at this stage), suggesting that no major changes occurred in the synthesis or turnover of the DAT protein in this phase of cell growth. The experiments reported in this work were done with cells harvested at a density between 5 and 7×10^5 cells/cm^2.

With a sucrose-containing phosphate buffer as used by many investigators of the dopamine transporter [40, 49, 50], Boja and colleagues [49] observed two binding site populations (or states) for [^{3}H]WIN 35,428 in COS cells transfected with cDNA encoding the rat DAT; the high-affinity component was similar to the binding population reported here in terms of K_d and B_{max}, although the expression system (COS versus C6) and the species (rat versus human) were different. Two binding components for [^{3}H]WIN 35,428 were also observed by Pristupa *et al.* [51] with a high Na^+, complex Tris/HEPES buffer in COS cells expressing the hDAT; only the high-affinity population was associated with dopamine uptake. So far, only one-site models have been reported for the binding of the closely related [^{125}I]RTI-55 ligand to cloned DATs, i.e. LLC-PK1 cells stably expressing the rat DAT (in NaCl-containing complex sodium phosphate buffer) [52], COS cells expressing the rat DAT (in phosphate buffer) [53], and C6 cells expressing the hDAT identical to the C6-hDAT used in the current study (in Krebs HEPES buffer) [54]. In view of the potential heterogeneity of ligand binding to the hDAT, we performed an initial series of experiments under the conditions of the protection protocol examining binding of a low concentration of [^{3}H]WIN 35,428 (0.45 nM) with an extended range of unlabeled WIN 35,428 (from 0.4 up to 1000 nM) in assays with larger volumes, as we described previously for rat striatal membranes [38]. Only one site was preferred by LIGAND with a K_d value of 10.6 ± 0.4 nM and a B_{max} value of 4.1 ± 0.1 pmol/mg protein (mean ± SEM for 3 independent experiments, data not shown). When these experiments were repeated wtih a sucrose (0.32 M)-containing phosphate buffer, again monophasic binding was observed. It is possible that the expression of the low-affinity component of either [^{3}H]WIN 35,428 or [^{125}I]RTI-55 binding in clones depends on the expression system and as yet undefined experimental conditions, although factors such as assay buffer (sucrose, ions) or species (rodent versus

TABLE 1. Saturation analysis of [^{3}H]WIN 35,428 binding and inhibition by *d*-amphetamine after pretreatment in the presence or absence of 6 nM NEM

Drug	Pretreatment	$K_{d,i}$ (M)	B_{max} (pmol/mg protein)
WIN 35,428	Control	$12.7 \pm 0.8 \times 10^{-9}$	2.16 ± 0.72
WIN 35,428	NEM	$9.9 \pm 1.4 \times 10^{-9}$	0.94 ± 0.05
d-Amphetamine	Control	$3.2 \pm 0.07 \times 10^{-6}$	
d-Amphetamine	NEM	$3.0 \pm 0.2 \times 10^{-6}$	

Membranes were pretreated with or without NEM as described in Materials and Methods. For other details, see the legend to Fig. 2. The K_d for WIN 35,428 was calculated by LIGAND, and the K_i for *d*-amphetamine was converted from its IC_{50} value by the Cheng–Prusoff equation [47]. Values are means ± SEM of three independent experiments carried out in triplicate. In the absence of inhibitor, the average specific binding of [^{3}H]WIN 35,428 was 328 ± 38 fmol/mg protein at a 2.6 nM concentration of radioligand.

monkey/human) do not seem to offer easy explanations. In this context, it remains to be demonstrated that the full hDAT transporter is expressed in the present C6 glioma cells. Although the extensive pharmacological profile reported for this clone expressed in C6 glioma cells [44] agrees with that expected for a dopamine uptake site, the actual gene product in the cells needs to be confirmed.

NEM-Induced Reduction of [^{3}H]WIN 35,428 Binding; Choice of Conditions

NEM was less potent in reducing [^{3}H]WIN 35,428 binding to C6-hDAT membranes than generally reported for radioligand binding to brain membranes [17, 33, 34, 55]. This seems to be related to the clone as expressed in the environment of the C6 glioma cell because parallel experiments with rat striatal membranes in our laboratory showed NEM to be significantly more potent under identical conditions of buffer, pH, and temperature (manuscript in preparation) (see also discussion below). The choice of the current high Na^+ containing buffer was based on the protection experiments by Heron *et al.* [34], Saadouni *et al.* [56] and Johnson *et al.* [33] who used a similar medium (100–120 mM NaCl and bicarbonate or HEPES for buffering); the high Na^+ medium was reported to retain binding of radioligand to the DAT better than the low Na^+ medium throughout the multiple centrifugation steps needed to wash out the NEM and protecting compound [56]. Under the present conditions, the compounds reported on in this work washed out satisfactorily, and complete protection was observed at the highest concentrations tested. However, our attempts to study GBR 12909 were hampered by the fact that this compound (at 200 nM) could not be removed, and even when an extra wash was carried out, the non-alkylated wash-out controls displayed levels of [^{3}H]WIN 35,428 binding far below that observed in samples treated identically without exposure to drug (75% reduction). This is in agreement with the findings of Rothman *et al.* [57] and Saadouni *et al.* [56], making it hard to study this type of blocker in protection studies.

Lack of Effect of NEM on Affinity Displayed by Remaining Uptake Sites Towards WIN 35,428 or d-amphetamine

It was important to assess whether NEM treatment eliminated [^{3}H]WIN 35,428 binding sites without somehow altering the remaining sites. To this end, membranes were pretreated in the presence or absence of 6 mM NEM under the same conditions as normally applied in the protection experiments. Because previous experiments with extended ranges of WIN 35,428 did not uncover more than one binding site (see above), saturation analysis was limited to measurement of this site. The observed lack of effect on the K_d value for [^{3}H]WIN 35,428 binding along with a marked reduction in B_{max} is consonant with NEM accomplishing essentially a loss of sites, in agreement with reported findings concerning NEM and binding of [^{3}H]methylphenidate [55] and [^{3}H]cocaine [56]. Pretreatment with NEM also did not affect the potency of *d*-amphetamine in inhibiting [^{3}H]WIN 35,428 binding, an important observation in the context of interpreting the lower protective potency of substrates as compared with blockers discussed below.

Protection against NEM: Divergence Between Blockers and Substrates, and Comparison with Other Studies

The present results extend those of Johnson *et al.* [33] who reported a divergence between cocaine, on the one hand, and dopamine and *d*-amphetamine, on the other hand, in protecting [^{3}H]mazindol binding sites against alkylation by NEM. An important additional piece of information that the present study provides is the inhibitory potency of the protecting compounds measured under the same conditions as those used for NEM treatment, allowing direct comparison of PC_{50} and IC_{50} values. In contrast, in the study by Johnson *et al.* [33], alkylation by NEM was performed at pH 8.0 which increases the affinity of cocaine-like inhibitors for the DAT [32]. This could have contributed to the observation that cocaine was relatively more potent than dopamine or *d*-amphetamine in protecting [^{3}H]mazindol

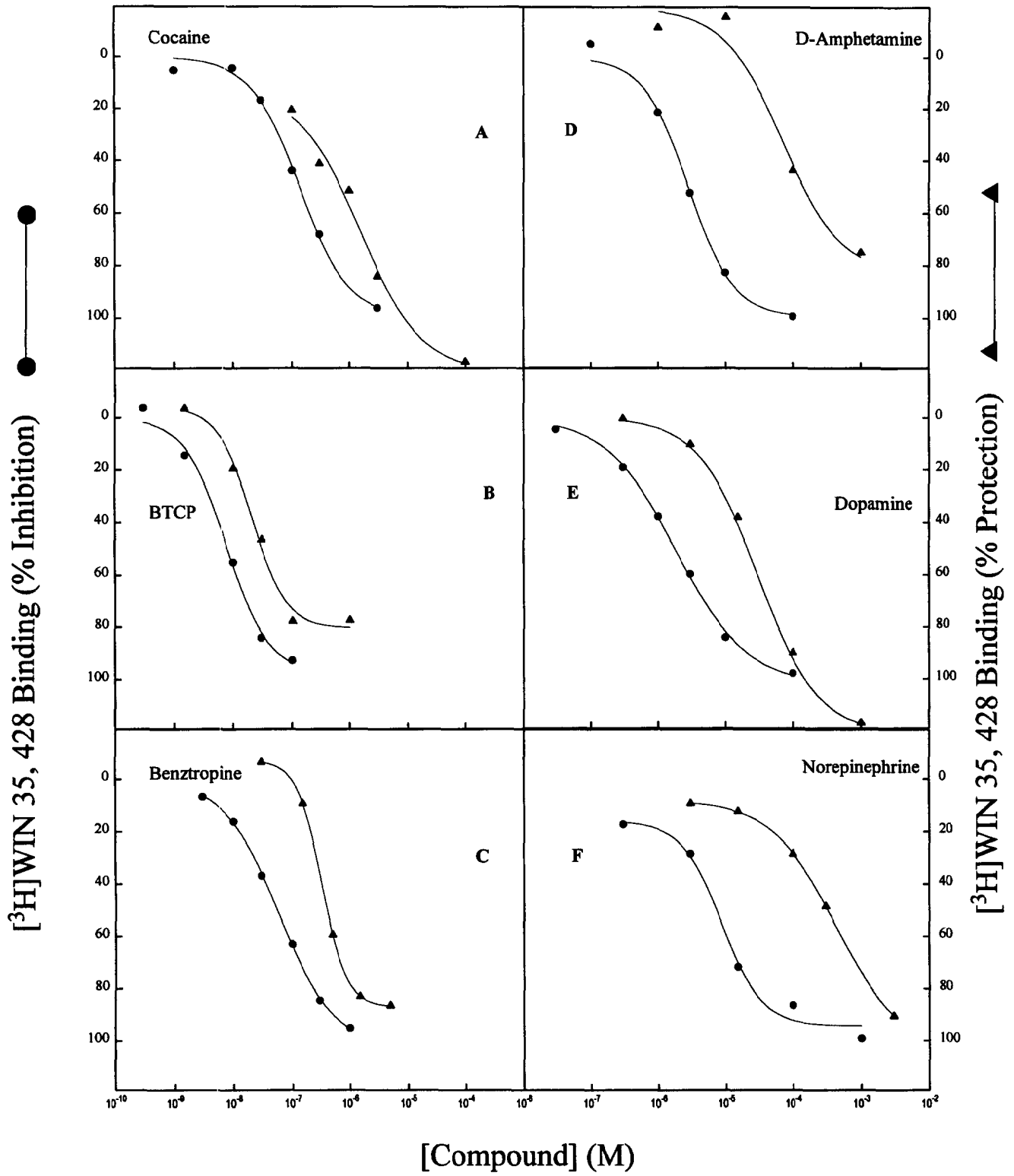

FIG. 3. Protection (▲—▲) against NEM-induced alkylation of [^{3}H]WIN 35,428 binding sites and inhibition (●—●) of [^{3}H]WIN 35,428 binding. In protection experiments, C6-hDAT membranes were preincubated with the test compound in sodium phosphate buffer containing 102 mM NaCl for 50 min, and subsequently exposed to 6 mM NEM. After removal of compound and NEM by repeated washing/centrifugation steps, binding of [^{3}H]WIN 35,428 was measured. Inhibition of [^{3}H]WIN 35,428 binding in the presence of the test compound was measured by incubating C6-hDAT membranes for 50 min under the same conditions as the protection experiments. For more information on the entire set of protection and inhibition experiments, and the levels of control specific binding in the absence of inhibitor, see Table 2.

TABLE 2. Potency of compounds in protecting against NEM-induced alkylation of [^{3}H]WIN 35,428 binding sites (PC_{50}) and in inhibiting [^{3}H]WIN 35,428 binding (IC_{50})

Drug	PC_{50} (M)	Hill	IC_{50} (M)	Hill
BTCP	$32.6 \pm 5.8 \times 10^{-9}$ (3)	2.16 ± 0.72 (3)	$8.7 \pm 0.59 \times 10^{-9}$ (3)	1.12 ± 0.07 (3)
Cocaine	$568.4 \pm 1.7 \times 10^{-9}$ (3)	0.94 ± 0.05 (3)	$113.8 \pm 22.1 \times 10^{-9}$ (4)	1.12 ± 0.14 (4)
Benztropine	$550.5 \pm 104.5 \times 10^{-9}$ (3)	1.61 ± 0.32 (3)	$48.6 \pm 3.9 \times 10^{-9}$ (3)	0.95 ± 0.03 (3)
Dopamine	$39.2 \pm 3.1 \times 10^{-6}$ (3)	0.89 ± 0.08 (3)	$1.7 \pm 0.1 \times 10^{-6}$ (3)	0.80 ± 0.06 (3)
d-Amphetamine	$81.3 \pm 33.8 \times 10^{-6}$ (3)	0.67 ± 0.12 (3)	$2.3 \pm 0.18 \times 10^{-6}$ (3)	1.09 ± 0.13 (3)
Norepinephrine	$288.2 \pm 53.9 \times 10^{-6}$ (3)	0.99 ± 0.12 (3)	$6.6 \pm 0.35 \times 10^{-6}$ (3)	0.76 ± 0.03* (3)

The protecting and inhibiting potency of compounds was determined as described in Materials and Methods. Values are means ± SEM of the number of independent experiments indicated in parentheses. In the absence of protecting compound, the average specific binding of [^{3}H]WIN 35,428 was 892 ± 89 fmol/mg protein at a 4.3 nM concentration of radioligand; in the inhibition experiments, this value was 90 ± 7 fmol/mg protein at a 0.45 nM concentration of radioligand.

* $P < 0.05$ compared with unity (single-sample *t*-test).

binding sites against NEM attack [33], as pointed out by Heron *et al.* [34], especially as the pH dependency of dopamine for the DAT may be opposite (lower affinity at higher pH) to that of cocaine analogs [30]. However, the present results confirm that substrates such as dopamine or *d*-amphetamine are less potent in protecting the DAT against alkylation by NEM than cocaine according to a direct comparison of PC_{50} and IC_{50} values (ratios of 23 or 35 vs 5). In contrast to the current results, Heron *et al.* [34] observed no difference between cocaine, on the one hand, and *d*-amphetamine or dopamine, on the other hand, in the ratio of PC_{50} over IC_{50} values for protection against NEM-induced reduction of [^{3}H]GBR 12783 binding to the DAT. This is most likely related to the different radioligands used to label the DAT, [^{3}H]WIN 35,428 and [^{3}H]GBR 12783, and it is consonant with the suggestion that cocaine and GBR 12783 recognize nonidentical but overlapping binding domains [56, 58]. Although the results from our previous experiments could be explained without invoking nonidentical binding domains for WIN 35,428 and another analog of the GBR series, GBR 12935 [17, 29], the existence of mutually exclusive binding does not rule out the involvement of nonidentical binding domains (see Reith *et al.* [29]), and competition experiments over a limited range of inhibitor concentrations may not reveal the special case of hyperbolic competitive inhibition (equivalent to negative allosterism) [59, 60]. The lack of a differential effect of NEM on [^{3}H]WIN 35,428 and [^{3}H]GBR 12935 binding in our previous work [17] as opposed to the divergent effect on [^{3}H]cocaine and [^{3}H]GBR 12783 binding in the study by Saadouni *et al.* [56] may be due to the use of low versus high Na^+-containing media. Also, in agreement with the involvement of different binding domains in the interaction of cocaine- and GBR-like compounds with the DAT is the observation of Pristupa *et al.* [51] that COS cells expressing the hDAT display [^{3}H]WIN 35,428 but not [^{3}H]GBR 12935 binding with the familiar pharmacological profile.

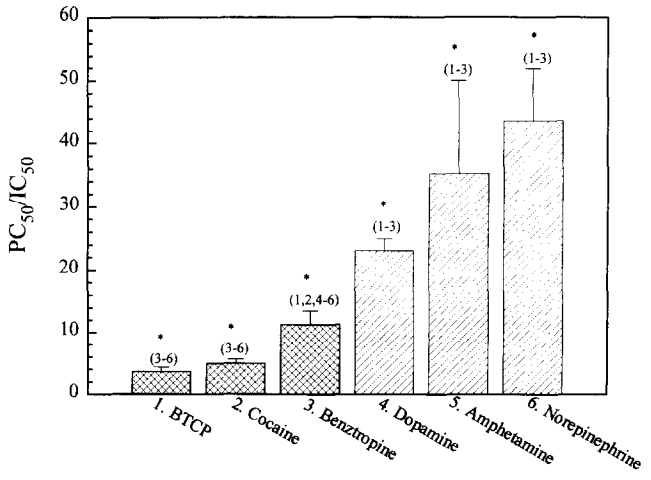

FIG. 4. Ratio of protective and inhibitory potency of compounds in the NEM alkylation experiments. All experimental details are as in Table 2. Values are means ± SEM of 3–4 independent experiments. Key: (*)$P < 0.05$ compared with the numbered conditions indicated between parentheses (one-way ANOVA followed by the Least Significance Difference Multiple Range test).

Protection against NEM: Why $PC_{50} > IC_{50}$?

In the present study, all compounds (not only substrates) had higher PC_{50} than IC_{50} values. A relatively higher concentration required for protection, as compared with inhibition, was also observed by Heron *et al.* [34]. The latter report describes how this can be exacerbated by addition of NEM and protecting blocker (not substrate) at the same time, suggesting the requirement of some time for the development of the inhibitory effect of blockers (as opposed to that of the faster interacting substrates). However, it can be calculated that the binding of blockers (and substrates) to the available binding sites has easily reached equilibrium within the 50-min preincubation period used in this study (see Xu *et al.* [39]). Most likely, the general mismatch between PC_{50} and IC_{50} values results from the interaction between an *irreversible* alkylator, NEM, and a *reversible* inhibitor. Although the mass action law applies to the extent that increasing concentrations of protecting compound are needed at higher NEM levels [33, 34] and that compounds with a lower affinity for the DAT are weaker protectors

(Heron *et al.* [34] and current study), absolute PC_{50} and IC_{50} values can be conceptualized as being different: as the NEM reaction progresses, binding sites are being removed from a pool susceptible to protection and the concentration of remaining free sites dwindles. At the same time, the protector is in the process of re-establishing equilibrium with the unreacted sites, opening up sites for alkylation [for instance, if the protector is present at its IC_{50} and after 50% of all sites have been alkylated, re-establishment of equilibrium would open up 50% of the unreacted sites, i.e. 25% of all sites, for alkylation; after some time, when 50% of those sites (12.5% of total) have reacted, a new equilibrium would make 6.25% of the total sites available for alkylation; and so on]. It can be understood, therefore, that alkylation will proceed as a function of exposure time to NEM, and that a virtually full occupancy of sites by the protector is needed to prevent the irreversible action of NEM.

Protection against NEM; Kinetic Considerations for the Difference between Substrates and Blockers

Because NEM is an irreversible inhibitor, one could think that there is no equilibrium between NEM and the reactive sites on the protein, which would further complicate the interaction between NEM and protector. However, as pointed out by Brocklehurst [61], the general debate in the literature on this issue was resolved in favor of the description of time-dependent protein modification by reagents such as NEM as composed of an equilibrium between reagent, protein, and reagent–protein complex as well as a step in which the complex is converted into the modified protein. A convincing case has been made that the second-order rate constant governing the latter conversion (k_{+2}) is likely to be much smaller than that (k_{-1}) for the dissociation of the reagent–protein complex in the equilibrium reaction when the rate constant for the protein modification reaction is much smaller than the association rate constant of 10^7 M^{-1} s^{-1} (or greater) commonly observed for complex formations (for references see Brocklehurst [61]). When NEM and the protector are present at the same time, the reaction between the protector and the domains on the transporter protein containing the NEM-reactive sites will not be at equilibrium if the rate constant (k) that governs the overall conversion of protein to NEM-alkylated end product is not appreciably smaller than the association rate constant (k_{+3}) of the reaction between protein and protector. It could be speculated that substrates have a higher PC_{50}/IC_{50} ratio than blockers because their k_{+3} values are small enough so that the alkylation process interferes with the substrate–protein equilibrium rendering the substrate a less potent protector. However, the k_{+3} value of dopamine, 1.4×10^6 M^{-1} s^{-1} as estimated by Meiergerd and Schenk [62], is close to that of cocaine estimated at 3×10^6 M^{-1} s^{-1} from its K_d of 109 nM and k_{-3} of 0.35 s^{-1} [63], and both of these values far exceed the estimate of 3×10^4 M^{-1} s^{-1} for the k value for NEM alkylation [61]. In addition, based on this line of reasoning, the blocker BTCP with an estimated k_{+3} of 4×10^4 M^{-1} s^{-1} (calculated from its K_d of 8.3 nM and k_{-3} of 0.00037 s^{-1} [64]) would be expected to be relatively less potent as a protector, a result that was not observed. It appears that under the present conditions with a 50-min preincubation period with protector, the differences between substrates and blockers cannot be explained by kinetic considerations.

A Conformational Model for the Different Protective Potency of Blockers and Substrates

The difference between blockers and substrates in protecting against NEM-induced alkylation of the hDAT can be interpreted in different ways. According to the model of Bonnet and co-workers [34, 41, 56], blockers bind with a change in enthalpy consistent with a conformational change of the DAT as opposed to the increase in entropy observed upon binding of substrates, suggesting hydrophobic links; the blocker-induced conformational change could make NEM-reactive residues inaccessible to NEM even though these sites may not be themselves involved in the interaction between the inhibitor and the DAT. Although this certainly is a possibility, it should be emphasized that the evidence for the lack of a conformational change upon binding of substrates is indirect. The thermodynamic analysis, as pointed out in the original study of Bonnet *et al.* [41], is theoretically only valid if the inhibitor binds to the same site as the radioligand, which is still open to debate [5, 23, 25, 26, 29, 30, 33]. A recent extension of the one-site substrate/blocker model has been advanced by Heron *et al.* [34] with the addition of a slow transition step from the transporter-blocker complex to a second conformationally different transporter-blocker complex. The model is consistent with the thermodynamic data but awaits experimental validation.

Different Protective Potency of Blockers and Substrates as Accommodated by a Model Involving Different Binding Domains

Alternatively, the differential effects of blockers and substrates in the NEM experiments can be viewed as a consequence of the involvement of different binding domains [33]. At the pH used in the present study, NEM interacts mostly with the thiol group of cysteine (see Schweri [55]), a residue that occurs in 12 different positions in the DAT [1–3]. Possibly, one of these residues (site 1) is involved in the binding of both a blocker such as cocaine and a substrate such as dopamine; a second site (2) is exclusively involved in cocaine binding, whereas the binding of radioligand WIN 35,428 requires both sites 1 and 2. In this situation, cocaine will be able to protect [^{3}H]WIN 35,428 binding against NEM attack, whereas dopamine will not because NEM can still alkylate site 2. This model is similar to that of Johnson *et al.* [33] except for the radioligand ([^{3}H]WIN 35,428 vs [^{3}H]mazindol) and NEM reactivity (one vs two classes of NEM sites). As far as the radioligands

are concerned, common binding domains are definitely involved in the binding of WIN 35,428 and mazindol [17, 29], but additional contribution of nonidentical domains has also been suggested based on recent results from rotating disk voltammetric experiments with striatal suspensions [65]. In the present study with C6-hDAT membranes, higher concentrations of NEM were needed for inactivation of hDAT radioligand binding than with rat striatal membranes; perhaps the conformation of the cloned hDAT in this expression system has rendered site 1, the more reactive NEM site involved in both cocaine and dopamine binding in the model of Johnson *et al.* [33], less reactive towards NEM alkylation. An additional explanation is needed for the protection that was observed in the present experiments with very high concentrations of substrates at PC_{50}/IC_{50} ratios of 23 and over, as opposed to the ratios of 4 to 5 seen with cocaine and BTCP. Perhaps dopamine, when bound, partially extends into the domain containing NEM site 2 so that NEM can still reach its site but now has a lower affinity (smaller chance of a chemical hit); this conversion to a lower-reactive NEM site is then seen as "protection" in our experiments.

Potential Positions of NEM Sites in the DAT Protein in the Above Model Involving Different Binding Domains for Blockers and Substrates

Because information about blocker and substrate domains on the DAT from mutagenesis and chimera studies has just begun to develop recently, one can only speculate on potential locations of the NEM-sensitive sites. The above-mentioned site 1 (involved in the binding of both cocaine and dopamine) could be Cys^{90} at the border of transmembrane domain 1 in the vicinity of Asp^{79} which has been proposed to be involved in the interaction with both cocaine and dopamine [25]. Site 2 (exclusively involved in cocaine binding) could be Cys^{305} in extracellular loop 3, Cys^{318} in transmembrane domain 6, or Cys^{341} in intracellular loop 3, which would be consonant with the results from chimeric dopamine-norepinephrine transporters, indicating the importance of the central domains (encompassing transmembrane domains 6–8 from extracellular loop 3 through intracellular loop 4) for interactions with cocaine and antidepressants [28]. Although the above-mentioned residues Cys^{90}, Cys^{305}, and Cys^{341} are among those that could be mutated to Ala without impacting the ligand binding or uptake activity of the DAT, as shown in a recent study by Uhl and co-workers [66], this does not rule out their involvement in NEM-induced reductions in binding activity. Thus, the substitution of one amino acid for another (Cys) is not equivalent to adding (by chemical linkage) an NEM molecule to the Cys residue. It would be of interest to investigate the susceptibility of the Cys-mutated DATs to NEM.

Concluding Remarks

The present results for benztropine are interesting in view of the recent observation of Meltzer *et al.* [67] that the S-enantiomers of 2-carbomethoxy-benztropine fluorinated analogs were more potent than the respective R-enantiomers in inhibiting [^{3}H]WIN 35,428 binding in contrast to the well-known lower potency of S-cocaine as compared with R-cocaine. Thus, benztropine and cocaine may bind to the same general domain of the DAT but do so in a different conformation, or their binding may involve different domains. Both of these possibilities are consonant with a different involvement of an NEM-sensitive site in the binding interaction of benztropine and cocaine. As far as the second possibility is concerned, the report of Meiergerd and Schenk [65] of a competitive interaction between benztropine and cocaine in inhibiting dopamine uptake, as measured by rotating disk electrode voltammetry, suggests the involvement of a common binding domain in addition to nonidentical domains.

In further defining the interaction between blockers or substrates and the DAT, it is important to combine the results of various experimental approaches, because subtle differences in binding interactions may be detected by one but not necessarily another technique. Because mutation and chimera cloning designs can potentially raise questions regarding activity changes due to insertion effects or conformational alterations remote from the mutated/chimeric sites, other approaches not relying on changes in the DAT itself remain important in providing complementary information.

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