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TRANSLOCATION OF DOPAMINE AND BINDING OF 2 β-CARBOMETHOXY-3 β-(4-FLUOROPHENYL) TROPANE (WIN 35,428) MEASURED UNDER IDENTICAL CONDITIONS IN RAT STRIATAL SYNAPTOSOMAL PREPARATIONS

INHIBITION BY VARIOUS BLOCKERS

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Abstract—Translocation of [^{3}H]dopamine and binding of 2 β-carbomethoxy-3 β-(4-fluorophenyl)[^{3}H]-tropane ([^{3}H]WIN 35,428) were measured in crude synaptosomal preparations from rat striatum under identical conditions of assay buffer (phosphate-Krebs) and temperature (25°). [^{3}H]Dopamine uptake as a function of time was close to linear for at least 8 min, whereas [^{3}H]WIN 35,428 binding had reached equilibrium within 1 min and remained at its plateau value for at least 20 min. The following inhibitors were tested in uptake and binding assays run in parallel with the same synaptosomal preparation: cocaine, WIN 35,428, benztropine, nomifensine, mazindol, methylphenidate, *N*-[1-(2-benzo[b]-thiophenyl)cyclohexyl]piperidine (BTCP), Lu 19-005 (Indatraline), 1-(2-(di(4-fluorophenyl)-methoxy)-ethyl)-4-(3-phenyl-2-propyl)piperazine (GBR 12909), 1-(2-(diphenylmethoxy)-ethyl)-4-(3-phenyl-2-propyl)piperazine (GBR 12935) and 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline (CGS 12066B). When present together with [^{3}H]dopamine or [^{3}H]WIN 35,428 for 8 min, the observed binding IC_{50} values were generally higher (average 1.4-fold) than the uptake IC_{50} values, with a significant y-axis intercept in linear regression analysis of binding on uptake IC_{50}. For slowly equilibrating inhibitors, estimates of uptake IC_{50} values were overestimates, and relatively lower values were obtained by monitoring [^{3}H]dopamine uptake for 1 min only during the last minute of the 8-min presence of inhibitor; under these conditions, binding over uptake IC_{50} ratios were on the average 2.3. Kinetic calculations, taking into account both radioligand and inhibitor equilibration kinetics, indicated that the latter comparison between binding and uptake measurements was most relevant, and suggested the involvement of complexities beyond simple competitive inhibition of dopamine transport, such as different binding domains for substrate and blocker recognition, or spare receptors for blockers. The present data indicate that binding over uptake IC_{50} ratios should be interpreted with caution, depending on the experimental conditions used to measure these ratios.

Key words: dopamine transporter; dopamine translocation; WIN 35,428 binding; equilibration kinetics; rat striatum

There is considerable evidence linking the rewarding and reinforcing effects of cocaine with its ability to block neuronal uptake of dopamine [1, 2]. Several earlier reports describe a competitive mechanism for the inhibition of dopamine uptake by cocaine and many other dopamine uptake blockers [3, 4], consonant with a common, mutually exclusive binding domain for blockers and substrates. However, a number of recent observations suggest a more complex model. First, site-directed mutagenesis of dopamine transporters has produced not only mutants that are more deficient in translocating [^{3}H]dopamine than in binding the phenyltropane analog of cocaine, [^{3}H]WIN 35,428† [5], but also mutants that translocate [^{3}H]dopamine normally but are deficient in [^{3}H]WIN 35,428 binding [6]. Second, the substrates dopamine and amphetamine offer

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† Abbreviations: BTCP, *N*-[1-(2-benzo[b]thiophenyl)-cyclohexyl]piperidine; CGS 12066B, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline; GBR 12783, 1-(2-(diphenylmethoxy)-ethyl)-4-(3-phenyl-2-propenyl)piperazine; GBR 12909, 1-(2-(di(4-fluorophenyl)-methoxy)-ethyl)-4-(3-phenyl-2-propyl)piperazine; GBR 12935, 1-(2-(diphenylmethoxy)-ethyl)-4-(3-phenyl-2-propyl) piperazine; Lu 19-005, Indatraline; WIN 35,428, 2 β-carbomethoxy-3 β-(4-fluorophenyl) tropane; k_{-1}, dissociation rate constant; and k_1, association rate constant.

substantially less protection than the blocker cocaine against alkylation of [^{3}H]mazindol binding sites on the dopamine transporter by the sulfhydryl reagent *N*-ethylmaleimide [7]. Third, binding of [^{3}H]BTCP to the dopamine uptake complex is reduced by dopamine via a mechanism other than competitive inhibition [8]. Fourth, a thermodynamic analysis of [^{3}H]mazindol and [^{3}H]GBR 12783 binding suggests that the binding of substrates is entropy-driven (hydrophobic), whereas the binding of inhibitors is generally enthalpy-driven (conformational change) [9]. Fifth, when dopamine uptake is measured on a time frame of seconds in striatal suspensions by rotating disk voltammetry, cocaine is an uncompetitive inhibitor possibly acting at the Na^+ binding site [10]. All these observations are consonant with the view that different binding domains are involved in the interaction of substrates and blockers with the dopamine transporter, perhaps in addition to a shared or overlapping domain. This is important, because it allows for a putative cocaine antagonist that, without itself blocking dopamine uptake, interferes with the action of cocaine. Two potential cocaine antagonists have been reported: GBR 12909, which attenuates the ability of cocaine to elevate extracellular dopamine in the striatum [11], and (7α-methoxy)cocaine, which weakens the potency of cocaine in inhibiting dopamine uptake [12].

In searching for a cocaine antagonist, one possible strategy is to look for a compound that potently inhibits binding of a cocaine-related radioligand (or a ligand of which the binding involves some of the same domains) to the dopamine transporter but does not affect dopamine translocation. The conditions commonly used for these assays have been arrived at by optimizing the activity to be measured. Thus, ligand binding and dopamine translocation are generally monitored under very different conditions, even though it is well known that the interaction of many compounds with the dopamine transporter depends highly on variables such as temperature [9, 13] and assay medium [14–22]. A notable exception is a recent report of Rothman *et al.* [23] on the inhibition of [^{125}I]RTI-55 [2 β-carbomethoxy-3 β-(4-[^{125}iodo]phenyl)tropane] binding and [^{3}H]-dopamine uptake by WIN 35,428, GBR 12935, and RTI-55 under the same conditions. In the present study, identical conditions were adopted for the measurement of the binding of [^{3}H]WIN 35,428 and the translocation of [^{3}H]dopamine in crude synaptosomal preparations from rat striatum, and the inhibition was assessed by several dopamine uptake blockers with widely different chemical structures including the putative cocaine antagonist GBR 12909 [11]. A greater potency of a compound in inhibiting binding as compared with uptake, under otherwise identical conditions, could indicate the involvement of different binding domains in the action of that compound and dopamine.

MATERIALS AND METHODS

Materials. [^{3}H]WIN 35,428 (82.4 Ci/mmol) and [^{3}H]dopamine (33.25 Ci/mmol) were obtained from Dupont-New England Nuclear (Boston, MA, U.S.A.). Cocaine hydrochloride was from the Mallinckrodt Chemical Corp. (St. Louis, MO, U.S.A.). GBR 12909 and WIN 35,428 were from Research Biochemicals Inc. (Natick, MA, U.S.A.). Methylphenidate was from the Research Triangle Institute (Research Triangle Park, NC, U.S.A.). Lu 19-005 was a gift from Lundbeck (Copenhagen, Denmark), mazindol from Sandoz (Basel, Switzerland), and nomifensine from Hoechst-Roussel Pharmaceuticals Inc. (Somerville, NJ, U.S.A.). BTCP and GBR 12935 were synthesized by Drs. Brian de Costa, Kenner C. Rice and A. E. Jacobson (NIDDK, NIH, Bethesda, MD, U.S.A.). All other chemicals were from Sigma (St. Louis, MO, U.S.A.) or Fisher (Springfield, NJ, U.S.A.). GBR compounds were dissolved in dimethyl sulfoxide by gentle warming (approximately 3 min) and sonicating (2 min); then water was added to give a 10% (v/v) dimethyl sulfoxide solution. Dilutions were made in 10% (v/v) dimethyl sulfoxide containing 0.01% (w/v) bovine serum albumin (intended to minimize adsorption to the walls of tubes); the final concentration of dimethyl sulfoxide in the assays was 0.5% (v/v) or less and did not by itself affect [^{3}H]WIN 35,428 binding or [^{3}H]dopamine uptake.

Animals and synaptosomal preparation. Male Sprague–Dawley rats (250–350 g, 2–3 months of age) were derived from a breeding colony located in Peoria, IL, U.S.A. Striatal tissue was dissected and homogenized in 15 vol. of ice-cold 0.32 M sucrose in a glass homogenizer with a motor-driven Teflon pestle (7 strokes up and down at 800 rpm). The homogenizer and pestle were rinsed with 30 vol. of 0.32 M sucrose. This fluid and the homogenate were combined and centrifuged at 1,000 *g* for 10 min at 0–4°. The supernatant fraction was centrifuged subsequently at 17,000 *g* for 20 min. Unless indicated otherwise, the resulting pellet (P_2) was homogenized in approximately 0.04 mL of 0.32 M sucrose per mg of initial tissue weight with a glass–teflon homogenizer; part of this homogenate was diluted 2-fold for the uptake experiments (see below).

Binding of [^{3}H]*WIN* 35,428 *and uptake of* [^{3}H]-*dopamine under identical conditions ("standard conditions").* Binding and uptake assays were performed (every condition in triplicate) in parallel on the same P_2 preparation. Binding assays were carried out in a total volume of 0.2 mL in 1-mL ministrip tubes (Skatron, Sterling, VA), and uptake assays in a total volume of 0.4 mL in borosilicate culture tubes (12 × 75 mm). The final concentrations of all components, in both binding and uptake assays, were: 73 mM NaCl, 3 mM KCl, 0.7 mM $MgSO_4$, 6 mM glucose, 0.6 mM $CaCl_2$, 0.006 mM nialamide, 0.08 M sucrose, 18 mM Na^+ and 10 mM phosphate from a mixture of primary and secondary phosphate buffer (giving a pH of 7.4 at room temperature), approximately 0.25 mg (for binding) or 0.12 mg (for uptake) of P_2 protein per mL assay medium, and test drug (a total of 8 concentrations evenly spaced around the IC_{50}). The binding assays contained, in addition, [^{3}H]WIN 35,428 at 3.6 nM, and the uptake assays included [^{3}H]dopamine consisting of 4 nM radiolabeled plus 46 nM unlabeled dopamine. For uptake (binding) measurements, the tubes were filled with 240 (120) μL buffer stock and 20 (10) μL test drug and kept on ice. Approximately

10 min prior to initiating the assay, the tubes were transferred to a waterbath at 25°. Approximately 0.5 min prior to starting the uptake (binding) assay, 40 (20) μL radioligand stock was added, and at zero time 100 (50) μL P_2 suspension was aliquotted followed by gentle vortexing. The assay mixture was incubated at 25° for 8 min with the shaker set at 50 rpm; the reaction was terminated by the addition of an excess of ice-cold wash buffer (73 mM NaCl, 3 mM KCl, 0.7 mM $MgSO_4$, 6 mM glucose, 0.6 mM $CaCl_2$, 30 mM Na^+ and 17 mM phosphate from a mixture of primary and secondary sodium phosphate, giving a pH of 7.4 at room temperature) and filtration over Whatman GF/F glass fiber filters with a single-manifold Millipore filtration apparatus (uptake) or Skatron receptor binding filtermats (glass fiber filter, 1 μm retention, No. 11734, equivalent to Whatman GF/B) with a mini-harvesting apparatus (type 11021, Skatron) (binding). All filters were pretreated with 0.05% (w/v) poly-L-lysine. After the first filtration, filters were washed 3 times with 4(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, U.S.A.) fluid]. Nonspecific uptake or binding was defined with 100 μM cocaine. Nonspecific uptake (or binding) was 4 (or 3) % of the total value determined in the absence of inhibitor. Protein content was estimated by the Folin phenol reagent method as described previously [16].

In a second approach for measuring [^{3}H]dopamine uptake (also done in parallel with binding), the experiments were worked up in sets of 24 assay mixtures for harvesting in the Brandel cell harvester with Whatman GF/C filters. Incubations with drug and [^{3}H]dopamine were started with less than 5 sec between samples. Immediately after the addition of ice-cold stop solution on the same time schedule, all 24 samples were filtered collectively. This procedure results in a variable waiting time (0–2 min) between adding the stop solution and filtering the mixture, but in control experiments (data not shown) it was found that there was no loss of accumulated [^{3}H]-dopamine taken up during waiting times of up to 6 min, the longest interval tested. With GF/C filters the filtration by the Brandel harvester was more rapid than with GF/F filters, and the uptake results were the same.

Binding of [^{3}H]*WIN* 35,428 *and uptake of* [^{3}H]-*dopamine: saturation studies.* Saturation analysis of [^{3}H]WIN 35,428 binding was performed on the same data used for calculating the IC_{50} of WIN 35,428 in inhibiting binding. The concentration of [^{3}H]WIN 35,428 was 3.6 nM, and the varying concentrations of added unlabeled WIN 35,428 were 0, 1.5, 5, 15, 30, 50, 100, 150, 300, and 500 nM. For saturation analysis of [^{3}H]dopamine uptake, the concentration of [^{3}H]dopamine was 4 nM, and the varying concentrations of added unlabeled dopamine were 0, 30, 100, 300, 1,000, 3,000 and 10,000 nM. Nonspecific binding or uptake was defined with 100 μM cocaine.

Binding of [^{3}H]WIN 35,428 *and uptake of [^{3}H]-dopamine: Time–course studies.* All procedures were as outlined above (standard conditions), with the following exceptions. In the experiments without test drugs, incubations were terminated at varying times between 1 and 40 min. In the case of [^{3}H]WIN 35,428 binding, a comparison was made between, on the one hand, mixing P_2 membranes suspended in 0.32 M sucrose with a buffer stock solution resulting in the final concentrations as detailed above and, on the other hand, mixing membranes suspended in buffer containing the final concentrations of all components with buffer stock solution at the same final concentrations.

In one series of experiments, the inhibition of [^{3}H]dopamine uptake by a given compound was studied during an uptake period of both 1 and 8 min in parallel experiments on the same membrane preparation. In both cases, the compound was present from 8 min before termination of the assay (i.e. in the 1-min assay, cocaine and P_2 membranes were added 7 min prior to [^{3}H]dopamine). In another series of experiments, the 1-min uptake assay was run in parallel with the [^{3}H]WIN 35,428 binding assay with the same membrane preparation.

Binding of [^{3}H]*WIN* 35,428: *inhibition and dissociation studies under various conditions.* In one line of experiments, the inhibition by mazindol was studied under conditions used in our previous studies [24, 25] for measuring [^{3}H]WIN 35,428 binding. For this purpose, P_2 membranes were resuspended in 25 mM sodium phosphate buffer (48 mM Na^+, pH 7.7, at 0–4°), and binding assays were conducted in the same buffer for 2 hr at 0–4°.

For the measurement of the dissociation rate, [^{3}H]WIN 35,428 binding was allowed to establish itself for 8 min under the standard conditions described above. Measurement of the dissociation rate was initiated by the addition of WIN 35,428 to a final concentration of 1 μM, in the presence or absence of 5 μM Lu 19-005. The disappearance of specifically bound radioactivity in time was determined by filtering samples at 0, 15, 30, 50, and 60 sec.

In another set of dissociation experiments, [^{3}H]-WIN 35,428 binding was allowed to establish itself for 8 min in the presence or absence of Lu 19-005 close to its IC_{50} concentration (10–20 nM). Subsequently, measurement of the dissociation rate was initiated by the addition of WIN 35,428 to a final concentration of 1 μM, in the presence or absence of 10 nM Lu 19-005. The disappearance of specifically bound radioactivity in time was determined as described above.

Data analysis. The IC_{50} values and pseudo-Hill numbers were computed with the equation of the ALLFIT program of De Lean *et al.* [26] entered into the Microsoft ORIGIN curve-fitting and plotting software. This nonlinear regression program was run with total and nonspecific uptake (binding) entered as constants. Equilibrium binding data were analyzed with the nonlinear computer fitting program LIGAND [27]. 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. Dissociation rates were calculated with the KINETIC module that is part of the same RADLIG package that contains LIGAND (Elsevier-Biosoft,

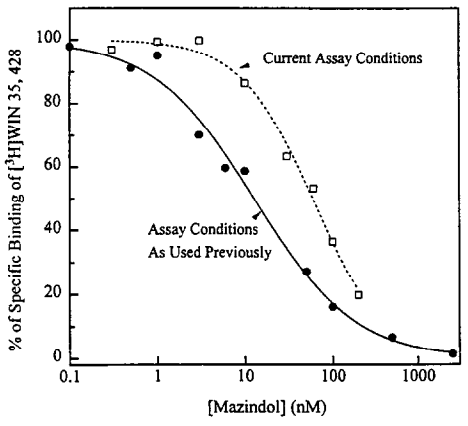

Fig. 1. Inhibition of [^{3}H]WIN 35,428 binding by mazindol. The assays were performed under the current standard conditions (□--□) or with 25 mM sodium phosphate buffer (2 hr on ice) (●——●). Points shown are those obtained in a single experiment, assayed in triplicate, that was carried out three times with the same results (see text). The control binding in the absence of mazindol was 0.60 pmol/mg of protein for the standard condition, and 0.72 pmol/mg of protein with 25 mM sodium phosphate as the buffer.

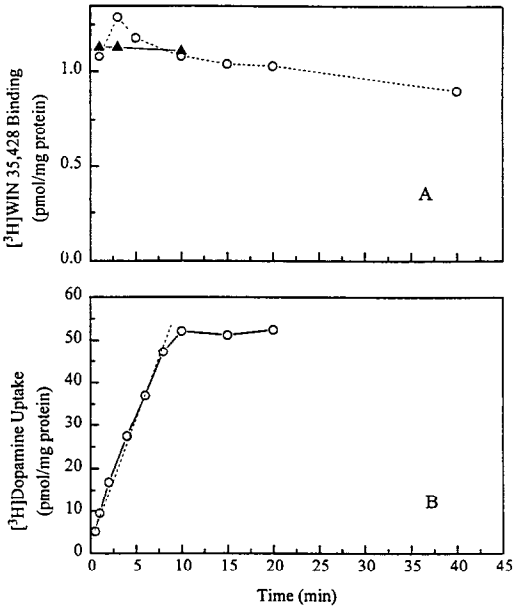

Fig. 2. Time-course of [^{3}H]WIN 35,428 binding (A) and [^{3}H]dopamine uptake (B) under standard conditions. (A) At time zero, either membranes suspended in 0.32 M sucrose were mixed with buffer stocks resulting in the final standard concentrations (○), or membranes suspended in buffer containing all final standard concentrations were mixed with buffer stocks containing the same final concentrations (▲). (B) At time zero, membranes suspended in 0.32 M sucrose were mixed with buffer stocks resulting in the final standard concentrations (○). The broken straight line represents the result of least squares linear regression of the data between 0 and 8 min. Points shown are those obtained in a single experiment assayed in triplicate that was carried out two times with the same results.

Cambridge, U.K.). Statistical analyses included linear least-squares regression analysis, correlation analysis, Student's one sample *t*-test, and the Wilcoxon signed-rank test. The accepted level of significance was 0.05. The discussed binding over uptake IC_{50} ratios from the study by Dr. L. Toll and colleagues (Neuroscience Department, Stanford Research Institute International, Menlo Park, CA) were obtained in the NIDA Drugs Medication Program; more details are available from Ms. A. Reid, NIDA, Medications Development Division, Room 11A-55, 5600 Fishers Lane, Rockville, MD 20857.

RESULTS

Characteristics of binding and uptake under standard conditions. As an example of the impact of conditions on [^{3}H]WIN 35,428 binding to P_2 membranes, inhibition by mazindol under the current standard assay conditions (25°, 8 min, complex sodium phosphate buffer) was compared with that assessed under conditions as used in our previous studies (0–4°, 2 hr, buffer consisting of 25 mM sodium phosphate only) (Fig. 1). The IC_{50} value shifted from 41 ± 9 nM (mean ± SEM for 3 independent preparations) to 15 ± 3 nM (mean ± 0.5 × range for 2 independent preparations). Although the affinity of mazindol, like that of WIN 35,428, was lower under the present standard conditions than under conditions developed previously for obtaining optimal binding data, the goal of the present study was to study binding under conditions suitable for measuring dopamine translocation simultaneously.

Under the standard conditions chosen for this study, [^{3}H]WIN 35,428 binding reached plateau levels within 1 min, and stayed essentially constant between 10 and 40 min (Fig. 2A). The time-course for P_2 membranes initially resuspended in 0.32 M sucrose, prior to mixing with the assay medium containing the buffer ingredients and [^{3}H]WIN 35,428, showed a small peak of binding at 3 min (dotted curve in Fig. 2A). In the latter experiments, the membranes were exposed to a change from 0.32 M sucrose to the final assay concentrations of all buffer components while the association of [^{3}H]-WIN 35,428 binding was ongoing. In contrast, when this change was avoided by resuspending the membranes in buffer containing the final concentrations of all components, the time-course of binding between 1 and 10 min was completely flat (solid curve in Fig. 2A). At the 10-min point, there was no difference in binding between membranes resuspended originally in 0.32 M sucrose or buffer containing all buffer components at the final assay concentrations.

Uptake of [^{3}H]dopamine under the standard conditions was close to linear with time for at least

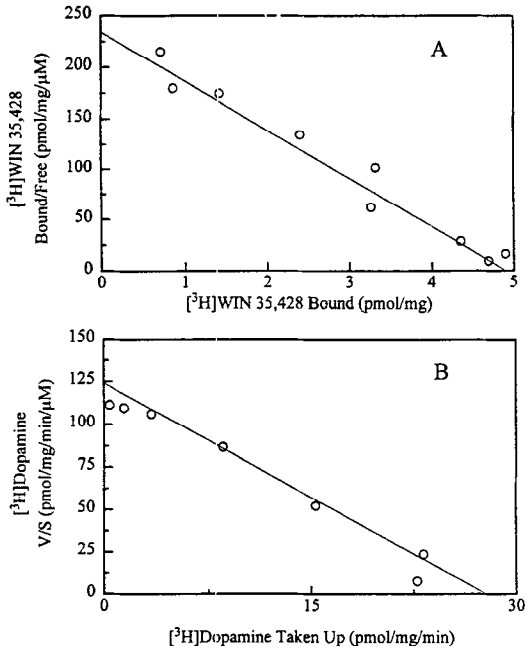

Fig. 3. Saturation analysis of [^{3}H]WIN 35,428 binding (A) and [^{3}H]dopamine uptake (B) under standard conditions. (**A**) [^{3}H]WIN 35,428 was present at 3.6 nM and increasing concentrations of unlabeled WIN 35,428 were added up to 500 nM. (**B**) [^{3}H]Dopamine uptake was present at 4 nM and increasing concentrations of unlabeled dopamine were added up to 10,000 nM. Nonspecific binding or uptake was defined with 100 μM cocaine. The straight line represents the best fit chosen by the LIGAND program. Shown is a typical experiment, assayed in triplicate, that was carried out 4 (A) or 2 (B) times (see text).

8 min (Fig. 2B). This made it possible to carry out uptake experiments with an interval of 8 min, at which time [^{3}H]WIN 35,428 binding was still at its plateau level (Fig. 2A), instead of an interval of 1 min as used in our previous studies [16].

Saturation analysis of [^{3}H]WIN 35,428 binding equilibrated for 8 min yielded a monophasic Scatchard plot (Fig. 3A), and a two-site fit solution could not be reached by the program LIGAND. The estimated K_d was 24 ± 3 nM and the B_{max} was 5.7 ± 0.5 pmol/mg of protein (mean ± SEM for four independent preparations). Similarly, the Eadie–Hofstee plot of [^{3}H]dopamine uptake was monophasic (Fig. 3B), and a two-site solution could not be arrived at by LIGAND. The estimated K_m was 160 ± 52 nM and the V_{max} was 22 ± 6 pmol/mg of protein/min (mean ± 0.5 × range for two independent preparations).

Effect of blockers on binding and uptake under standard conditions (8 min). The Hill numbers estimated by the ALLFIT program for inhibition of [^{3}H]WIN 35,428 binding under the present standard conditions were close to unity for the majority of the inhibition curves (Table 1). Exceptions were the curves for Lu 19-005, GBR 12909, GBR 12935, and CGS 12066B, which were relatively steep with Hill numbers greater than unity. For the inhibition of [^{3}H]dopamine uptake, there were no differences between the Hill numbers obtained with the standard 8-min uptake time and those observed with a 1-min uptake time; therefore, those values were pooled (Table 1). Again, Hill numbers greater than unity were observed for Lu 19-005, GBR 12909, GBR 12935, and CGS 12066B; in addition, the inhibition curves of BTCP were steep.

When studied under standard conditions with 8 min for both [^{3}H]WIN 35,428 binding and [^{3}H]-dopamine uptake, the inhibitory potencies of Lu 19-005, GBR 12909, and GBR 12935 (No. 8, 9, and 10, non-circled data points in Fig. 4 with numbers corresponding to those in Table 1) showed appreciable variation between days with binding and uptake IC_{50} values changing in tandem. Because the binding and uptake experiments were run in parallel with the same membrane preparation and the same drug stocks, this variation could be thought to be related to drug stability or solubility. As far as the latter possibility is concerned, there might be different amounts of dissolved drug in the stock solutions, a common problem with these rather water-insoluble, lipophilic compounds that tend to be adsorbed onto the walls of tubes. It appears to be related to the compounds and not the assays, because none of the other drugs tested (No. 1, 2, 3, 4, 5, 6, 7, 11) showed this variation. When the binding and uptake inhibitory potencies, obtained pairwise on the same experimental day with the same drug stocks, were plotted in a correlation diagram (Fig. 4, non-circled numbers), the observed IC_{50} values for inhibiting binding and uptake were close to the theoretical line describing a perfect one-to-one ratio (solid straight line) or above this line reflecting binding over uptake ratios greater than unity. The greatest deviations from the perfect one-to-one relationship were noted at low inhibitor concentrations (see broken straight regression line in Fig. 4 for all non-circled data points) with ratios close to 2. Linear regression analysis on all non-circled data points together indicated an IC_{50} value of 18 nM for inhibition of [^{3}H]WIN 35,428 binding at an IC_{50} of 10 nM for inhibition of [^{3}H]dopamine uptake. The 95% confidence interval of the former value was 15–21 nM (computed by intercept interval analysis with standard linear regression techniques), well above the 10 nM value expected from a perfect one-to-one binding over uptake ratio ($P < 0.001$, two-tailed confidence interval analysis).

Effect of blockers on binding (8 min) and uptake (1 min). The possibility was considered that the 8-min [^{3}H]dopamine uptake assay gives an overestimate of the IC_{50} value in cases where the inhibitor needs time to occupy its sites while dopamine uptake is going on, resulting in relatively less inhibition in the early phase of the assay. In parallel experiments with the same membrane preparation, shortening of the uptake time from 8 to 1 min (while keeping the total time with inhibitor at 8 min) had little or no effect on the inhibition curve of cocaine (Fig. 5A), but resulted in a shift to the left of the inhibition curve of BTCP (Fig. 5B). For cocaine, the IC_{50} was 255 ± 24 nM (8 min) as compared with 191 ± 28 nM (1 min) (mean ± SEM for 3 independent experiments) ($P > 0.05$ paired Student's t-test). In contrast,

Table 1. Hill numbers for inhibition of binding of [^{3}H]WIN 35,428 and uptake of [^{3}H]dopamine

	[^{3}H]WIN 35,428 binding	[^{3}H]Dopamine uptake
1. Cocaine	0.90 ± 0.04 (5)	0.86 ± 0.08 (5)
2. WIN 35,428	0.94 ± 0.06 (4)	1.04 ± 0.06 (4)
3. Benztropine	1.13 ± 0.14 (3)	1.11 ± 0.04 (3)
4. Nomifensine	1.05 ± 0.18 (3)	0.92 ± 0.11 (3)
5. Mazindol	1.13 ± 0.03 (3)	1.07 ± 0.06 (3)
6. Methylphenidate	0.91 ± 0.09 (3)	1.03 ± 0.09 (3)
7. BTCP	1.12 ± 0.10 (3)	1.63 ± 0.09† (3)
8. Lu 19-005	1.43 ± 0.12* (3)	1.67 ± 0.03† (3)
9. GBR 12909	3.70 ± 0.92† (5)	3.20 ± 0.63† (5)
10. GBR 12935	2.05 ± 0.48* (4)	1.65 ± 0.09† (3)
11. CGS 12066B	1.60 ± 0.15* (3)	2.13 ± 0.18† (3)

P_2 membranes from rat striatum were incubated with inhibitor and 3.6 nM [^{3}H]WIN 35,428 (for binding, 8 min) or 50 nM [^{3}H]dopamine (for uptake, 1 or 8 min) at 25°. Nonspecific binding or uptake was defined with 100 μM cocaine. Values are means ± SEM of the number of independent experiments indicated in parentheses. In the absence of inhibitors, the average binding of [^{3}H]WIN 35,428 was 0.7 pmol/mg of protein; the average uptake of [^{3}H]-dopamine was 11 pmol/mg of protein for the 1-min assays, and 41 pmol/mg of protein for the 8-min assays.

* $P < 0.05$, one-sample Student's t-test (alternative hypothesis: Hill number is greater than unity).

† $P < 0.05$, one-sample Student's t-test (alternative hypothesis: Hill number is greater or smaller than unity).

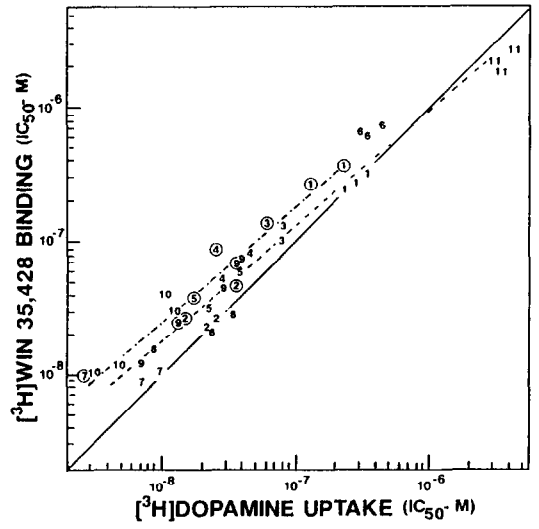

Fig. 4. The IC_{50} values of compounds in inhibiting [^{3}H]WIN 35,428 binding and [^{3}H]dopamine uptake under standard conditions. Each point represents a paired observation of both the binding and uptake IC_{50} obtained with the same membrane preparation and the same stocks of drugs. The numbering of the compounds is as in Table 1. The non-circled data were obtained with an 8-min period for both binding and uptake; the circled data were obtained with 8 min for binding and 1 min for uptake. The solid straight line represents a theoretical line describing a perfect one-to-one relationship between IC_{50} values for binding and uptake. The broken straight line represents the results of least squares linear regression analysis of all non-circled data ($r = 0.98$, $N = 29$, $P < 0.001$), and the broken-dotted straight line the linear regression results for all circled data ($r = 0.97$, $N = 10$, $P < 0.001$).

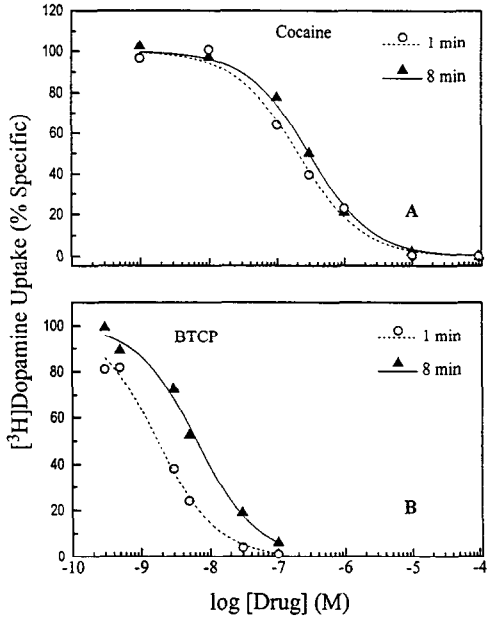

Fig. 5. Inhibition of [^{3}H]dopamine uptake by cocaine (A) and BTCP (B). The inhibitor was present for 8 min, and [^{3}H]dopamine was present for either 1 min (○--○) or 8 min (▲——▲). Points shown are those obtained in a single experiment, assayed in triplicate, that was carried out three times with the same results; the 1- and 8-min data were collected pairwise with the same membrane preparation. The average control uptake in the absence of inhibitor was 10 pmol/mg of protein after 1 min and 51 pmol/mg of protein after 8 min. The Hill numbers at 1 and 8 min were, for cocaine, 0.92 and 1.11 (top panel); and for BTCP, 1.00 and 1.00 (bottom panel).

for BTCP the shift in the inhibition curve was more than 2-fold with the IC_{50} falling from 7.7 ± 0.8 nM to 3.1 ± 0.7 nM (mean ± SEM for 3 independent experiments) ($P < 0.05$, paired Student's *t*-test).

In consonance, when paired IC_{50} values, obtained in parallel experiments with 8 min for binding and 1 min for uptake, were compared with those obtained under the standard conditions of 8 min for both binding and uptake, a general shift to the left was noted in the correlation diagram (Fig. 4, compare the circled and non-circled numbers for compounds 1, 3, 4, 5, and 7). The ratio of binding (8 min) over uptake (1 min) IC_{50} values was again greater than unity at low inhibitor concentrations with ratios as high as 3 (broken-dotted straight line in Fig. 4 for all circled data points). Linear regression analysis on all circled data points together indicated an IC_{50} value of 24 nM for inhibition of [^{3}H]WIN 35,428 binding at an IC_{50} of 10 nM for inhibition of [^{3}H]-dopamine uptake. The 95% confidence interval of the former value was 18.1 to 31 nM (computed by intercept interval analysis with standard linear regression techniques), well above the 10 nM value expected from a perfect one-to-one binding over uptake ratio ($P < 0.001$, two-tailed confidence interval analysis). For the 7 compounds that were tested at both 8- and 1-min uptake times (No. 1, 2, 3, 4, 5, 7, and 9), the binding over uptake IC_{50} ratio increased from 1.39 ± 0.16 (8-min uptake) to 2.28 ± 0.29 (1-min uptake) (average ± SEM for all compounds together) ($P < 0.05$, Wilcoxon signed-ranks test).

Dissociation rate of [^{3}H]*WIN* 35,428 *binding.* The high Hill number observed in some experiments with compounds such as Lu 19-005 raised the possibility that the mechanism of inhibition deviated from the competitive model. The following experiments addressed a potential allosteric effect of Lu 19-005 on the dissociation rate of [^{3}H]WIN 35,428 binding; such an allosteric effect is not expected for a competitive inhibitor in experiments in which the off-rate of previously equilibrated radioligand binding is measured [14, 24]. When the measurement of the dissociation of [^{3}H]WIN 35,428 binding was initiated by an excess (1 μM) of unlabeled WIN 35,428 in the presence or absence of 5 μM Lu 19-005, there was no effect of Lu 19-005 on the dissociation rate (Fig. 6A) (half-life of 16.9 ± 0.7 sec in the absence, and 14.8 ± 0.3 sec in the presence of Lu 19-005, average ± 0.5 × range of paired observations on 2 independent preparations assayed in triplicate). It is possible that this lack of effect is due to the fact that the dissociation rate of [^{3}H]WIN 35,428 binding is extremely rapid as compared with the time required for Lu 19-005 to interact with its binding sites. In a different approach, therefore, membranes were equilibrated with [^{3}H]WIN 35,428 with or without 10 nM Lu 19-005 (occupying approximately 50% of its binding sites), and the dissociation measurement was initiated by an excess of unlabeled WIN 35,428 (1 μM) in the absence or presence of 10 nM Lu 19-005 (Fig. 6B). Again, there was no effect of Lu 19-005 (half-life of 13.8 ± 0.2 sec in the absence, and 12.0 ± 1.4 sec in the presence of Lu 19-005, average ± 0.5 × range of paired observations on 2 independent preparations assayed in triplicate).

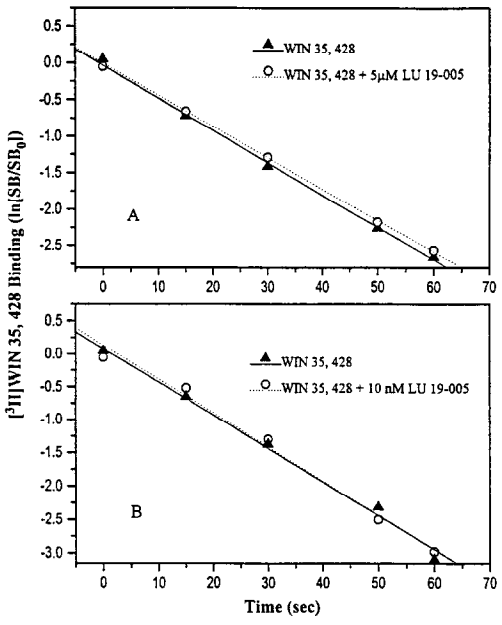

Fig. 6. Dissociation of [^{3}H]WIN 35,428 binding in the presence and absence of Lu 19-005. (**A**) [^{3}H]WIN 35,428 binding was established for 8 min, and at zero time measurement of dissociation was initiated by the addition of 1 μM unlabeled WIN 35,428 with (○--○) or without (▲——▲) 5 μM Lu 19-005. (**B**) [^{3}H]WIN 35,428 binding was established for 8 min with or without Lu 19-005 (10 nM final concentration), and at zero time measurement of dissociation was initiated by the addition of 1 μM unlabeled WIN 35,428 with (○--○) or without (▲——▲) 10 nM Lu 19-005. Data shown in each panel are from a single experiment, assayed in triplicate, that was carried out twice with similar results (see text). Straight lines represent the result of least squares linear regression. Specific binding (SB) at zero time (SB_0) was 0.81 (A) and 0.95 (B, without Lu 19-005) or 0.50 (B, with Lu 19-005) pmol/mg of protein.

DISCUSSION

Experimental conditions for measuring binding and uptake; kinetic considerations. Because assay conditions such as buffer, ionic environment, and temperature impact on the interaction between inhibitors and the dopamine transporter [9, 13, 15–23], care was taken in the present study to perform the binding and uptake assays under identical conditions. Initial experiments showed [^{3}H]WIN 35,428 binding to be very low at the physiological temperature of 37°, probably as a result of the unfavorable thermodynamics of radioligand binding (see also [13]) and the inhibitory effect of cations (K^+, Mg^{2+}, Ca^{2+}) (see [19]) present in the assay buffer; 25° was chosen as a compromise allowing reliable measurement of both binding and uptake. In choosing conditions, care was taken to preserve kinetic relevance, i.e. equilibration in the case of binding assays and initial velocity for uptake measurements. One would expect that the current 8-min incubation time is sufficient for [^{3}H]WIN 35,428 binding to equilibrate, considering that the

rate at which a radioligand, L, binds to its receptor depends on the term $e^{-K_A t}$ in the binding equation with a half-life of binding of $0.69/K_A$[28], in which $K_A = k_1[L] + k_{-1}$; in our case, [L] = 3.6 nM, appreciably lower than the K_d ($=k_{-1}/k_1$) (24 nM) of [^{3}H]WIN 35,428 binding, and therefore $K_A \sim k_{-1}$. After 5 half-lives, $3.5/K_A$ ($\sim 3.5/k_{-1}$), binding is only 3% less than the equilibrium value [28], which should be reached in the case of [^{3}H]WIN 35,428 binding (k_{-1} of 0.0481 sec^{-1} from a half-life of 14.4 sec, average of data in Fig. 6) in 3.5/0.0481 sec or 1.2 min. Indeed, plateau binding was observed in the shortest time point measured, 1 min (triangles, Fig. 2A). The small peak at 3 and 5 min observed for P_2 membranes resuspended in 0.32 M sucrose most likely represents the stimulatory effect of sucrose on [^{3}H]WIN 35,428 binding [21] during the initial time period after mixing the assay components when the membranes undergo a transition from an environment of 0.32 M sucrose to 0.08 M sucrose. It is possible that the same effect underlies the slight deviation from linearity observed in the plot of [^{3}H]-dopamine uptake against time (Fig. 2B). Be that as it may, the uptake of [^{3}H]dopamine under the current conditions leveled off after a much longer time than observed in our previous experiments [16]; most likely, this is due to the omission of oxygenation of the buffer and ascorbate in the current experiments, a combination now known to cause a loss of dopamine uptake capability associated with the generation of free radicals [29].

Although most receptor binding studies are performed with broken membrane preparations, plasma membrane dopamine transporters have been assayed for radioligand binding successfully in isotonically prepared P_2 fractions suspended in isotonic medium containing sucrose [17, 18, 30]. The binding sites are generally thought to face externally, and would thus be accessible in both broken and synaptosomal preparations; in addition, the interior milieu (high K^+) is not conducive to binding as opposed to the exterior (high Na^+) [see Refs. 19 and 21], making it likely that the binding we observe with synaptosomal preparations is to external sites [for the impact of preparative procedures see also Ref. 20].

The fact that the protein concentration in the [^{3}H]-WIN 35,428 binding assays was twice that in the [^{3}H]dopamine uptake assays has no impact on the measured IC_{50} values as long as the concentration of binding sites is substantially less than the equilibrium dissociation constant for the radioligand [31] (for a discussion of the impact of radioligand concentration see below). In the binding assays, the binding site concentration was 1.4 nM, appreciably less than the K_d (24 nM) for [^{3}H]WIN 35,428 binding. It can be calculated that in the absence of inhibitor approximately 0.18 nM of radioligand was bound, or 5% of totally available ligand, which was well within zone A [32]. Similarly, in the uptake assays, the binding site concentration was 0.7 nM, again considerably less than the K_m (160 nM) for [^{3}H]-dopamine uptake and well within zone A. The relatively higher protein concentration in the binding assays allowed an easily measurable level of bound radioactivity, whereas the relatively lower concentration in the uptake assays was necessary to restrict the depletion of medium [^{3}H]dopamine (by accumulation) to less than 10% of totally available substrate in the 8-min experiments; in the latter case, the accumulated radioactivity, at either 1 or 8 min, was always easily quantifiable. The purpose of raising the substrate concentration to approximately 50 nM by the addition of unlabeled dopamine in the uptake assays was to minimize the influence of the binding of dopamine to the transporter on the substrate concentration: if a one-to-one binding site ratio is assumed for [^{3}H]WIN 35,428 and dopamine (see below), depending on the equilibrium dissociation constant for dopamine binding, up to 0.7 nM could be removed from the medium by binding to the transporter.

The present conditions allow the performance of binding and uptake assays under identical conditions for an incubation period of 8 min with kinetic relevancy as far as the binding and uptake radioligands are concerned. Analysis of inhibition by a variety of compounds under these standard conditions (8 min) indicates binding IC_{50} values higher than the corresponding uptake IC_{50} values, with points forming a line displaced significantly (95% confidence interval analysis) compared with the line representing a perfect one-to-one ratio (Fig. 4). However, the IC_{50} estimates for uptake inhibition under the standard conditions (8 min) can, in some cases, be considered overestimates (causing an underestimate of the shift of the line compared with the "perfect" line in Fig. 4), because the situation for inhibitors, present along with radioligands, is more complex, as the following kinetic considerations show. If the dissociation rate constant for the binding of the inhibitor ($k_{-1,I}$) is much smaller than that of [^{3}H]WIN 35,428 binding ($k_{-1,WIN}$) (inhibitor binds more slowly than radioligand), the equilibration time of the entire system will depend on the time it takes the inhibitor to reach equilibrium with the free receptors ($1.75/k_{-1,I}$ at the IC_{50} concentration of inhibitor) [28]. After 8 min of co-presence of inhibitor and [^{3}H]WIN 35,428, we examine the system at that equilibrium or at its approach to that equilibrium. In contrast, in the 8-min [^{3}H]dopamine uptake experiments we allow [^{3}H]dopamine to be taken up in the early phase of the assay when the inhibitor has not yet had the chance to occupy its full potential of receptors, i.e. the uptake measure after 8 min reflects a summation of results of the different equilibration stages of the inhibitor binding as it approaches equilibrium. This is expected to have relatively more impact for compounds with a low $k_{-1,I}$; indeed, BTCP, which has a $k_{-1,I}$ of 0.00037 sec^{-1} (under conditions not entirely identical to the current ones) [33], showed a greater shift to the left in the concentration curve for uptake inhibition upon shortening the uptake time to 1 min (Fig. 5B) than cocaine, which has an extremely high $k_{-1,I}$ (0.029 sec^{-1}, determined under conditions not entirely identical to the current ones) [34] (Fig. 5A). Most compounds studied here bind quite rapidly, resulting in only small differences in IC_{50} values between the 1- and 8-min uptake assays; nevertheless, the combined 1-min data of the 7 compounds studied under both conditions lie on a line correlating

binding with uptake IC_{50} values that is shifted to the left compared with the line for the 8-min data (Fig. 4).

The equilibration of [^{3}H]dopamine itself can be expected to be extremely rapid. If dopamine binds to the dopamine transporter with an affinity of 15 μM (average, range 6–20 μM) as measured in binding experiments with [^{3}H]cocaine [35], [^{3}H]mazindol [16], or [^{3}H]GBR 12935 [36], and if an association rate of $10^6\ M^{-1}\ sec^{-1}$ is assumed, as observed for ligands binding to neurotransmitter receptors [32] (compare with $2.1 \times 10^6\ M^{-1}\ sec^{-1}$ for WIN 35,428 calculated from the present results), the $k_{-1,DA}$ for dopamine binding to the transporter is $15\ sec^{-1}$ corresponding to a half-life of 0.05 sec. Thus, in binding terms, [^{3}H]dopamine will be at equilibrium with the uptake carrier in a time frame ($3.5/k_{-1,DA}$) that is negligible compared with a 1- or 8-min uptake period, and it is only the equilibration time of the inhibitor (with a $k_{-1,I} \ll k_{-1,DA}$) that determines overall equilibration (see above). In the case where the inhibitor has a $k_{-1,I}$ that is greater than the $k_{-1,WIN}$ (an unlikely event because of the binding rapidity of [^{3}H]WIN 35,428) or greater than the $k_{-1,DA}$ (an extremely unlikely event), and if [L] $\ll K_d$ (true for WIN 35,428 binding {3.6 $\ll$ 24 nM}, and most likely for dopamine binding as well, see above {50 $\ll$ 15,000 nM}), equilibrium will be achieved (with the inhibitor present at its IC_{50} concentration) at $3.5/k_{-1}$ [28], i.e. 1.2 min for [^{3}H]WIN 35,428 binding and 0.2 sec for [^{3}H]dopamine binding. For such inhibitors, there is no difference between IC_{50} values determined in 1- and 8-min uptake assays, and the same results are expected in binding assays conducted for any length of time between 1.2 and 8 min.

For the inhibitors tested in the present study, $k_{-1,I}$ values are comparable to (case 1) or smaller than (case 2) the value of $k_{-1,WIN}$ and are likely smaller (both case 1 and case 2) than the $k_{-1,DA}$. In both cases, the degree of inhibitor equilibration will be comparable between binding assays with the inhibitor and [^{3}H]WIN 35,428 incubated together for 8 min, on the one hand, and uptake assays with the inhibitor present from time zero and [^{3}H]dopamine from time 7–8 min, on the other hand, for the following reasons. In case 1 ($k_{-1,I} \sim k_{-1,WIN} \ll k_{-1,DA}$), equilibration of the inhibitor (at its IC_{50} concentration) in the binding assay is reached between $1.75/k_{-1,WIN}$ and $3.5/k_{-1,WIN}$, or between 0.6 and 1.2 min, well within the total 8-min incubation period; likewise, in the uptake assay, the inhibitor (at its IC_{50} concentration) reaches equilibrium well within the first 7 min without dopamine being present ($1.75/k_{-1,WIN}$ or 0.6 min). In case 2 ($k_{-1,I} \ll k_{-1,WIN}$ and $k_{-1,I} \ll k_{-1,DA}$), the equilibration in the binding assay (at the IC_{50} concentration of the inhibitor) only depends on $k_{-1,I}$ and equals $1.75/k_{-1,I}$, which is identical to the equilibration time of the inhibitor by itself in the uptake assay during the initial 7 min without dopamine. In the latter case, [^{3}H]dopamine added at time 7 min (and present until the reaction is stopped at time 8 min) will equilibrate rapidly (high $k_{-1,DA}$) with the free sites, and because its concentration is much lower than $K_{d,DA}$ it will not appreciably shift the equilibrium between free and bound inhibitor. Therefore, under these conditions, the comparison between binding and uptake IC_{50} values (circled data in Fig. 4) is proper from a kinetic standpoint, giving final equilibrated values (case 1) or at least values at the same point (7–8 min) in the approach of inhibitor binding to equilibrium (case 2). In addition, the [^{3}H]dopamine uptake rate is still definitely linear with time under these conditions with a 1-min uptake interval (Fig. 2B).

Difference between binding and uptake inhibitory potency. Focusing on those compounds that were tested pairwise in both binding assays (8 min) and uptake assays (8 min for inhibitor, 1 min for [^{3}H]-dopamine) as described above (circled data in Fig. 4), we observed an average binding over uptake IC_{50} ratio of 2.28. Although this is not a large ratio considering the normal variation inherent to experiments of this kind, the fact that all compounds tested showed ratios consistently greater than unity is intriguing. Several possibilities can be entertained.

First, for competitive inhibitors, IC_{50} values are a function of the radioligand concentration L in comparison with the radioligand K_d or K_m: $IC_{50} = K_i(1 + \{L/K_{d\ or\ m}\})$ [37]. Applied to our conditions, for an inhibitor with equal binding and uptake inhibitory potency, this would cause a binding over uptake IC_{50} ratio of 0.88, not very different from unity and deviating in a direction opposite to that observed.

Second, it is possible that the inhibitors tested are substrates for the transporter allowing efflux of dopamine by reversed transport, or enter nerve terminals as lipophilic compounds and release vesicular dopamine that is subsequently transported outwardly by reversed transport, in an amphetamine-like fashion [38, 39]. Although some evidence supporting the latter possibility for GBR 12909 and 12935 has been advanced recently [40], it is not likely to be a unitary explanation that applies to all inhibitors tested in this study. For instance, cocaine which is not accumulated in brain slices at a tissue to medium ratio greater than unity [41], has a binding over uptake IC_{50} ratio close to 2 (No. 1 circled, Fig. 4); in addition, benztropine (No. 3), nomifensine (No. 4), mazindol (No. 5), and BTCP (No. 7), with ratios of 2 or more, do not show the dramatic dopamine efflux observed with amphetamine in microdialysis [42] or perfused slice [43] experiments.

A third possibility is that the binding domains for WIN 35,428 and dopamine include nonidentical portions, and therefore it could make a difference what is used as a ligand to radiolabel the transporter. In this scenario, one would expect WIN 35,428-like compounds such as WIN 35,428 itself, and cocaine, to be relatively more potent inhibitors of [^{3}H]WIN 35,428 binding, and other, more "dopamine-like" compounds to be more potent inhibitors of [^{3}H]-dopamine uptake. The present results, however, do not clearly place cocaine and WIN 35,428 (No. 1 and 2 circled, Fig. 4) in a category of relatively more potent binding inhibitors, with binding over uptake IC_{50} ratios of 1.5 and 1.7 as compared with an average of 2.3 for all compounds assayed under the paradigm of 8 min for inhibitor, 8 min for [^{3}H]WIN 35,428, and 1 min for [^{3}H]dopamine. To further address the possibility of the different binding domains, more compounds need to be tested under the present conditions.

Fourth, it is possible that a spare receptor reserve exists that allows a functional response to dopamine uptake blockers to occur at blocker concentrations lower than those required for binding to the transporter. The possible existence of spare receptors for blockers such as cocaine has not been advanced previously because assays for measuring binding (inhibition of radioligand binding) and response (inhibition of dopamine translocation) have not been performed in a comparable fashion, including both identical incubation conditions and kinetic relevance. Theoretically, the present data do not allow us to estimate the size of the receptor reserve, because the product of efficacy and receptor occupancy resulting in the response are inseparable: if the efficacy is sufficiently high, full inhibition can be obtained at low receptor occupancy [44]. However, the situation here is a transporter protein that translocates dopamine and is inhibited by blockers requiring no more than one molecule to block the transport of one molecule of dopamine as inferred from the monophasic inhibition curves observed for most compounds. Perhaps we can envision one spare receptor per transporter molecule; the rate-limiting process that causes the receptor reserve in the case of blocker-induced inhibition of the dopamine carrier could be the conformational changes that occur upon binding, such that changes induced by occupation of only one site are sufficient to immobilize the transporter, whereas radioligand binding experiments measure two sites per transporter. Alternatively, one could accommodate 2 sites for blockers if one functional transporter unit were a dimer consisting of 2 transporter molecules. From a B_{max} of 5.7 pmol/mg of protein for [^{3}H]WIN 35,428 binding, 1 or 2 sites per transporter, and a V_{max} of 22 pmol/mg of protein per min for [^{3}H]dopamine translocation, the turnover value of dopamine transport can be calculated as 0.06 or 0.13 sec^{-1} with first order half-lives of 11 or 5 sec. For compounds that are translocated, such as dopamine itself, the comparison between binding and uptake IC_{50} values is probably not valid because of another process in addition to binding site saturation that determines the saturation function of dopamine transport [16]. This other process could be a rate-limiting transporter reorientation step as suggested for serotonin [45] and norepinephrine [46] transport.

Fifth, we cannot rule out the possibility that there is some as yet unknown factor that makes compounds more potent in uptake assays than in binding assays even if these compounds are pure uptake blockers and not substrates. There may be some factor inherent in the difference between equilibrium binding and transport rate assays that needs to be taken into account.

Concluding remarks. The present observation of Hill numbers appreciably higher than unity for some compounds could indicate a mechanism of inhibition that deviates from competitive inhibition. For Lu 19-005 there was no allosteric effect on the dissociation rate of [^{3}H]WIN 35,428 binding (Fig. 6), but allosteric changes in the transporter complex that do not alter the dissociation rate cannot be excluded. In this context, it is of interest to note the high Hill numbers (1.3 to 3.0) reported by Deutsch *et al.* [47] for various newly synthesized GBR 12783 derivatives in inhibiting [^{3}H]methylphenidate binding and their suggestion for an underlying lack of equilibration. In addition, the extreme lipophilicity of some of the compounds with high Hill numbers may preclude careful kinetic analysis that requires precise knowledge of the ambient concentrations of the compound. The parallel analysis of binding and uptake, under the same conditions, with the same drug stocks, validates the comparison between binding and uptake IC_{50} values in the current study.

Binding over uptake IC_{50} ratios should be interpreted with caution depending on the experimental conditions used to measure these ratios. Compounds that have been identified previously as displaying low or high binding over uptake IC_{50} ratios with assays conducted under different conditions [48] are potentially interesting but need to be studied in more detail. As an example of the impact of conditions, the current results can be compared with those obtained in a study carried out by Dr. L. Toll and colleagues (Neuroscience Department, Stanford Research Institute International) as part of the NIDA Drugs Medication Program. In the latter study, [^{3}H]WIN 35,428 binding to rat striatal crude membranes was assayed in a 50 mM sodium phosphate buffer (100 mM NaCl) on ice, and [^{3}H]-dopamine uptake into P_2 fractions in a Krebs–HEPES buffer at 25°; the following binding over uptake IC_{50} ratios were observed: cocaine, 1.08; WIN 35,428, 1.71; nomifensine, 1.45; mazindol, 0.95; methylphenidate, 2.16; GBR 12909, 128; and CGS 12066B, 0.32. Clearly, both high (GBR 12909) and low (CGS 12066B) ratios have changed to values much closer to unity in the present study with binding and uptake assays conducted under identical experimental conditions. A similar conclusion was reached for WIN 35,428, GBR 12935, and RTI-55 by Rothman *et al.* [23] with [^{125}I]RTI-55 as the radioligand for binding. In the latter study, the inhibitor was preincubated with synaptosomes for 60 min at 25° prior to measurement of binding or uptake for an additional 20 min at 25°. This approach gives ample time for the inhibitor to equilibrate; although equilibration of [^{125}I]RTI-55 under these conditions is incomplete ($3.5/k_{-1}$ = 146 min with k_{-1} for the high-affinity binding site approximately 0.024 min^{-1} [49, 50]), the impact on the measured IC_{50} values is probably minor as long as the radioligand concentration is low enough such that the inhibitor binding equilibrium is not shifted appreciably. Be that as it may, all data taken together indicate the vital importance of experimental conditions in searching for a cocaine antagonist that interferes with cocaine binding to the dopamine transporter without itself inhibiting dopamine translocation.

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