0026-895X/93/020271-06$03.00/0

MOLECULAR PHARMACOLOGY, 43:271-276

Non-neurotoxic Amphetamine Derivatives Release Serotonin through Serotonin Transporters

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Received May 6, 1992; Accepted November 5, 1992

SUMMARY

3,4-Methylenedioxymethamphetamine (MDMA) and several other amphetamine derivatives cause degeneration of serotonergic nerve terminals. These drugs also release serotonin from nerve terminals both *in vivo* and *in vitro*. Two non-neurotoxic derivatives of MDMA were tested in membrane vesicle model systems to determine whether they also lacked the ability to release serotonin. 3-Methoxy-4-methylamphetamine (MMA) and 5-methoxy-6-methyl-2-aminoindan (MMAI) both inhibited imipramine binding to serotonin transporters in platelet plasma membrane vesicles and both inhibited Na^+ gradient-driven serotonin transport into those vesicles. Significantly, both MMA and MMAI released [^{3}H]serotonin from plasma membrane vesicles, apparently by a process of exchange. The half-maximal concentrations for this effect were comparable to that reported for MDMA. In addition to their effects on plasma membrane transporters, MMA and MMAI both inhibited serotonin transport into chromaffin granule membrane vesicles catalyzed by the vesicular biogenic amine transporter. At higher concentrations, these compounds also caused release of [^{3}H]serotonin from chromaffin granule membrane vesicles and dissipated the transmembrane pH difference (ΔpH). Although MMAI effects on the serotonin transporter were similar to those of MDMA, the two compounds had different effects on dopamine transporters. MDMA and methamphetamine inhibited binding of a cocaine analog to the dopamine transporter and released dopamine accumulated by cells expressing dopamine transporters, but similar concentrations of MMAI were inactive.

A class of amphetamine derivatives including MDMA, 3,4-methylenedioxyamphetamine, PCA, and fenfluramine cause degeneration of serotonergic nerve terminals (1–4). Each of these compounds releases serotonin by a nonexocytotic process. The nature of amphetamine-induced serotonin neurotoxicity is not understood, and the participation of serotonin release in the process has not been evaluated (5). Other potential mechanisms for amphetamine neurotoxicity involve an action on serotonergic terminals by released dopamine (6, 7), dissipation of ion gradients by transporter-mediated amphetamine cycling (8), and unspecified intracellular actions of the amphetamines or their metabolites (5).

Because MDMA has the property of releasing dopamine as well as serotonin, the role of released dopamine in the destruction of serotonergic terminals has been the subject of experimental scrutiny. The hypothesis that dopamine is involved in the toxicity of MDMA is supported by experiments demonstrating that dopamine receptor blockers prevent MDMA neurotoxicity (7). Nichols *et al.* (9) have synthesized analogs of MMDA that are not neurotoxic. MMAI and MMA both resemble MDMA in drug-discrimination trials (9) but do not by themselves destroy serotonergic terminals. Amphetamine itself is not a serotonergic neurotoxin, but amphetamine releases dopamine and its coadministration with MMAI causes the same long term loss of serotonin, 5-hydroxyindole acetic acid, and paroxetine binding activity as does MDMA (10). It was proposed that MMAI and MMA shared with MDMA the ability to release serotonin but lacked its effect on dopamine release and, therefore, did not lead to dopamine-mediated neurotoxicity.

We have recently developed membrane vesicle model systems in which to examine the effect of these amphetamine derivatives on serotonin transporters (8). A Na^+-dependent transporter is responsible for serotonin reuptake across the plasma membrane, and accumulation of cytoplasmic serotonin within secretory vesicles is coupled to exchange with intravesicular H^+ ions catalyzed by the vesicular amine transporter. In plasma membrane vesicles isolated from human platelets, MDMA and PCA stimulate release of internal [^{3}H]serotonin in a process mediated by the platelet plasma membrane serotonin transporter (8, 11). The value of platelets as a model for studying

This work was supported by a Grant-in-Aid from the American Heart Association and by United States Public Health Service Grant DA07259.

ABBREVIATIONS: MDMA, 3,4-methylenedioxymethamphetamine; PCA, *p*-chloroamphetamine; MMAI, 5-methoxy-6-methyl-2-aminoindan; MMA, 3-methoxy-4-methylamphetamine; β-[^{125}I]CIT, 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl)tropane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid).

neuronal serotonin transport has been recently validated by the isolation of cDNA clones coding for serotonin transporters from rat brain and rat basophilic leukemia cells (12, 13). The similarity between the two nucleotide sequences suggests that central and peripheral serotonin transporters are products of the same gene. Thus, agents that cause release of serotonin from platelet plasma membrane vesicles are also likely to release serotonin from nerve endings. Likewise, the chromaffin granule amine transporter is thought to provide a good model for serotonin accumulation within synaptic vesicles (14). With these two model systems we have been able to examine the mechanisms by which MDMA and PCA release serotonin from synaptic vesicles and nerve terminals (8, 11). In the work described here we tested the ability of MMA and MMAI to release serotonin in these membrane vesicle model systems.

Experimental Procedures

Materials. [^{3}H]Serotonin (12.6 Ci/mmol), [^{3}H]imipramine (40.4 Ci/mmol), and [^{3}H]dopamine (24.1 Ci/mmol) were purchased from New England Nuclear. MMA and MMAI were generous gifts from Dr. David Nichols, Purdue University. β-[^{125}I]CIT (2200 Ci/mmol) was synthesized as previously described for β-[^{123}I]CIT (15). It was a gift from Drs. Robert Innis, Yolanda Zea-Ponce, and Ronald Baldwin (Dept of Psychiatry, Yale University, and VA Medical Center, West Haven, CT), who prepared and characterized β-[^{125}I]CIT using the stannyl precursor of β-CIT synthesized by Drs. John Neumeyer and Richard Milius (Research Biochemicals, Inc., Natick, MA). All other reagents were reagent grade and were purchased from commercial sources.

Preparation of membranes. Outdated human platelet concentrates were purchased from the Connecticut Red Cross. Platelets from 50–100 individuals were pooled for each membrane preparation. Platelet plasma membrane vesicles were isolated by the method of Barber and Jamieson (16) with the modifications described previously (17). Chromaffin granule membrane vesicles were prepared as described by Schuldiner *et al.* (18), by repeated osmotic lysis of bovine adrenal medullary chromaffin granules isolated by differential sedimentation. Dissected rat striata were homogenized in 20 volumes of 150 mM Li_2SO_4 buffer containing 10 mM lithium borate, pH 8.0, and 1 mM $MgSO_4$, using a Brinkmann Polytron (Westbury, NY) (setting 6 for 30 sec). The homogenate was washed twice by centrifugation for 10 min at 50,000 × *g* and resuspension in the same buffer. The final membrane suspension was distributed into microcentrifuge tubes and sedimented by centrifugation. After the supernatant fluid was removed, the pellets were frozen and stored at −80° until use.

Binding assays. Imipramine binding was measured at 25° using the filtration assay described previously (19). Membrane vesicles were suspended at a protein concentration of 0.3 mg/ml in an assay buffer of 200 mM NaCl containing 10 mM lithium phosphate, pH 6.7, 1 mM $MgSO_4$, and the indicated concentration of [^{3}H]imipramine (19–23 cpm/fmol). After a 20-min incubation, the reactions (300 μl/assay) were terminated by dilution with 4 ml of ice-cold iso-osmotic NaCl and were filtered through Whatman GF/B filters that had been pretreated with 0.3% polyethyleneimine. The tube and filter were washed three times with 4 ml of ice-cold NaCl solution. Filters were placed in Optifluor (Packard, Downers Grove, IL) and counted after 5 hr. Binding in the absence of Na^+ or in the presence of 100 μM serotonin was taken as a control for nonspecific binding. The standard error of replicate assay values was typically <5% of the mean.

Transport assays. Transport of [^{3}H]serotonin into plasma membrane vesicles was measured at 25° using the previously described filtration assay (20). Unless otherwise indicated, vesicles were equilibrated with 10 mM lithium phosphate buffer, pH 6.7, containing 133 mM K_2SO_4 and 1 mM $MgSO_4$ and were diluted into external medium consisting of 0.2 M NaCl containing 10 mM lithium phosphate buffer, pH 6.7, 1 mM $MgSO_4$, and 0.1 μM [^{3}H]serotonin (12.3 Ci/mmol). Chromaffin granule membrane vesicles were diluted to a concentration of approximately 0.25 mg/ml in 0.3 M sucrose containing 10 mM potassium HEPES, pH 8.5, 5 mM KCl, 2.5 mM $MgSO_4$, 5 mM disodium ATP, and 0.1 μM [^{3}H]serotonin (unless otherwise indicated). Reactions (200 μl/assay) were stopped by dilution, filtration, and washing, and filtered vesicles were counted as described previously (18). The standard error of replicate assay values was typically <5% of the mean.

Exchange. Platelet plasma membrane vesicles equilibrated with 10 mM lithium phosphate buffer, pH 6.7, containing 60 mM NaCl, 93 mM K_2SO_4, and 1 mM $MgSO_4$ were diluted 30-fold into 0.2 M NaCl containing 10 mM lithium phosphate buffer, pH 6.7, and 0.1 μM [^{3}H]serotonin, at 25°. After serotonin accumulation had reached a maximum (5–10 min), efflux was initiated by dilution of the suspension 40-fold with the indicated medium. Thirty seconds after dilution, the vesicles were collected by filtration of the suspension through Gelman GN-6 nitrocellulose filters. The reaction tube and filter were rapidly rinsed with 2 ml of ice-cold 0.2 M NaCl and the filter was counted in 3 ml of Optifluor (Packard). Efflux from chromaffin granule membrane vesicles was measured in the same way, except that preloading was carried out in 0.3 M sucrose containing 10 mM potassium HEPES, pH 8.5, 5 mM KCl, 2.5 mM $MgSO_4$, 5 mM disodium ATP, and 0.1 μM [^{3}H]serotonin and efflux was measured after dilution into the same medium without ATP, $MgSO_4$, or [^{3}H]serotonin. The standard error of replicate assay values was typically <10% of the mean.

ΔpH measurements. Chromaffin granule membrane vesicles (80 μg of protein) were incubated at room temperature in a cuvette containing 2 ml of 10 mM Tris-EPPS (pH 8.5), 150 mM KCl, 6 μM acridine orange, and 5 mM ATP. The relative fluorescence of the mixture was measured with a Perkin-Elmer LS-5B luminescence spectrometer, using an excitation wavelength of 490 nm and an emission wavelength of 526 nm. When the base-line fluorescence stabilized, acidification was initiated by the addition of 6 mM $MgSO_4$ and the relative fluorescence was monitored. Approximately 15 min later, additions of MMAI or MMA were made and the increase in fluorescence was measured. After the last addition, NH_4Cl was added to a final concentration of 10 mM to neutralize the vesicle interior and completely reverse the quenching. In replicate experiments, the concentration of MMA and MMAI required for half-maximal reversal of the fluorescence quenching varied by <10% of the mean value.

Results

Plasma membrane serotonin transporter. To evaluate the interaction of MMA and MMAI with the plasma membrane serotonin transporter we used plasma membrane vesicles isolated from human platelets, which provide a good model system for studying this transporter (21). When vesicles equilibrated in K_2SO_4 are diluted into NaCl medium, the transmembrane gradients for Na^+, K^+, and Cl^- provide a driving force for accumulation of serotonin (20). Inhibitors, such as the tricyclic antidepressant imipramine, bind at or near the transporter substrate binding site and block serotonin transport (22).

Both MMA and MMAI inhibit [^{3}H]serotonin transport and [^{3}H]imipramine binding by platelet plasma membrane vesicles. The data in Fig. 1 show that, as described previously for MDMA (8), both MMA and MMAI inhibit serotonin transport. This inhibition is likely to result from direct interaction with the serotonin transporter, because both agents also inhibit [^{3}H]imipramine binding. Transport inhibition occurs at lower MMA and MMAI concentrations than are required to inhibit imipramine binding. In both assays, MMA is slightly more potent than MMAI.

We previously inferred that MDMA was a substrate for the transporter from its higher potency to inhibit transport, relative

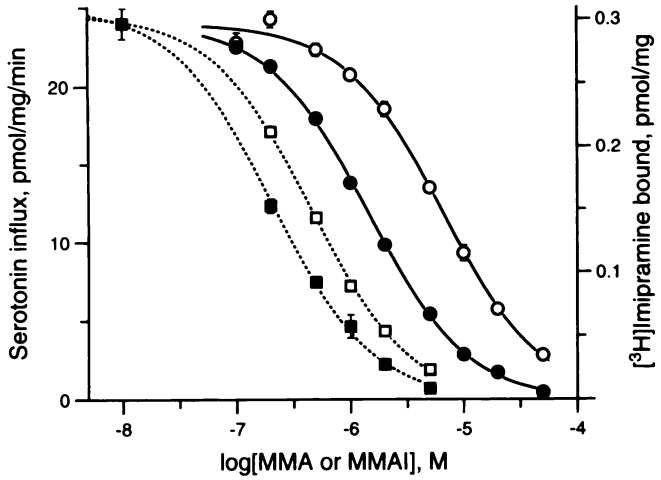

Fig. 1. Inhibition of platelet plasma membrane serotonin transport and imipramine binding. Rates of serotonin influx (– – –) were measured in 20-sec incubations as described in Experimental Procedures, using 0.1 μM [^{3}H]serotonin in the presence of the indicated concentrations of MMA (■) or MMAI (□). Uptake values due to nonspecific processes, measured in the presence of 1 μM imipramine, were subtracted from each measurement. Half-maximal inhibition of transport occured at 205 ± 15 nM MMA and 452 ± 18 nM MMAI. Imipramine binding (——) was measured as described in Experimental Procedures, using 1 nM [^{3}H]imipramine in the presence of the indicated concentrations of MMA (●) or MMAI (○). Nonspecific binding, measured in the presence of 100 μM serotonin, was subtracted from each measurement. Half-maximal inhibition of binding occured at 1.4 ± 0.45 μM MMA and 6.54 ± 0.65 μM MMAI. Each *point* represents the average of triplicate determinations. *Error bars* represent the standard deviation and are plotted only where they exceeded the size of the symbol.

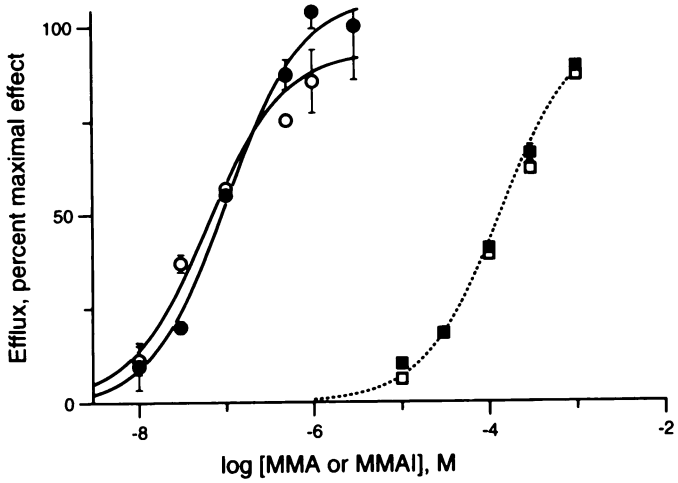

Fig. 2. Serotonin efflux from platelet plasma membrane vesicles and chromaffin granule membrane vesicles. Plasma membrane vesicles (——) that had accumulated [^{3}H]serotonin were diluted into NaCl medium containing the indicated concentration of MMA (○) or MMAI (●), and efflux was measured for 1 min as described in Experimental Procedures. The maximal extent of efflux was 55% in 1 min at 3 μM MMA. Half-maximal stimulation of efflux occurred at 61 ± 20 nM for MMA and 101 ± 20 nM for MMAI. Each *point* represents the average of triplicate determinations. *Error bars* represent the standard deviation and are plotted only where they exceeded the size of the symbol. Chromaffin granule membrane vesicles that had accumulated [^{3}H]serotonin were diluted into 0.3 M sucrose medium containing 10 mM potassium EPPS, pH 8.5, 2.5 mM $MgSO_4$, 5 mM KCl, and MMA (□) or MMA (■) at the indicated concentration, and efflux was measured for 10 min as described in Experimental Procedures (– – –). The maximal extent of efflux was 90% in 10 min at 1 mM with either compound. Half-maximal stimulation required 0.135 ± 0.021 mM concentrations of either compound. Each *point* represents the average of triplicate determinations. *Error bars* represent the standard deviation and are plotted only where they exceeded the size of the symbol.

to binding (8). This was confirmed by the ability of MDMA to exchange with internal [^{3}H]serotonin in a reaction catalyzed by the transporter. To test the ability of MMA and MMAI to exchange with serotonin, we allowed platelet plasma membrane vesicles to accumulate [^{3}H]serotonin and then diluted the vesicles into medium free of radiolabel. Previous results (23) indicate that external serotonin causes more rapid efflux of [^{3}H]serotonin by a rapid exchange reaction. In this partial reaction of the transporter, substrate is shuttled back and forth across the membrane (leading to exchange), avoiding a rate-limiting step in the net efflux reaction cycle. Neurotoxins that cause serotonin release *in vivo,* such as MDMA and PCA, also increased the rate of [^{3}H]serotonin efflux from membrane vesicles when present in the dilution medium (8, 11). The results in Fig. 2 demonstrate that MMA and MMAI accelerate release of [^{3}H]serotonin from preloaded vesicles. The concentrations required for efflux stimulation are similar to those required for inhibition of serotonin transport (Fig. 1), consistent with the notion that both phenomena result from MMA and MMAI acting as substrates for the serotonin transporter.

Increased efflux could result from many causes, including an increase in nonspecific membrane permeability. To conclude that MMA and MMAI induce efflux by acting as substrates for the transporter, it is necessary to demonstrate that efflux is blocked by a specific inhibitor of the serotonin transporter and that the process demonstrates the same ion dependence as transport. The results in Table 1 demonstrate that efflux caused by MMA and MMAI is inhibited by imipramine. Removal of external Na^+ leads to an increase in basal serotonin efflux (Table 1) (23). In the absence of external Na^+, however, the increase in efflux due to MMA and MMAI is dramatically inhibited. Thus, MMA- and MMAI-induced efflux is likely to be mediated by the serotonin transporter.

TABLE 1

Na^+ Dependence and Imipramine sensitivity of serotonin exchange with MMA and MMAI

Platelet plasma membrane vesicles equilibrated with medium containing 60 mM NaCl, as described in Experimental Procedures, were loaded with [^{3}H]serotonin and diluted into the following media: line 1, 0.2 M NaCl containing 10 mM lithium phosphate buffer, pH 6.7; line 2, 2 M imipramine added to control medium; line 3, NaCl replaced with equimolar LiCl. All values represent the percentage of [^{3}H] serotonin efflux in the first 30 sec after dilution. The vesicles contained 65 pmol of serotonin/mg of membrane protein (approximately 18 M) at the time of dilution.

	Efflux		
	Control	MMAI	MMA
	% of internal contents		
1. NaCl	20.2 ± 1.9	53.4 ± 2.1	54.3 ± 1.3
2. Imipramine	21.4 ± 1.3	23.4 ± 2.4	25.9 ± 0.6
3. LiCl	53.2 ± 1.8	56.4 ± 2.4	60.5 ± 1.1

Vesicular biogenic amine transporter. The accumulation of serotonin by chromaffin granule membrane vesicles is also inhibited by serotonin neurotoxins (8, 11). These vesicles represent a widely accepted model system for biogenic amine accumulation and storage within synaptic vesicles (14). Fig. 3 shows the results of an experiment in which MMA and MMAI were tested as inhibitors of [^{3}H]serotonin accumulation in chromaffin granule membrane vesicles. Both compounds are effective inhibitors of the vesicular amine transporter. The concentrations of these agents required to inhibit the vesicular

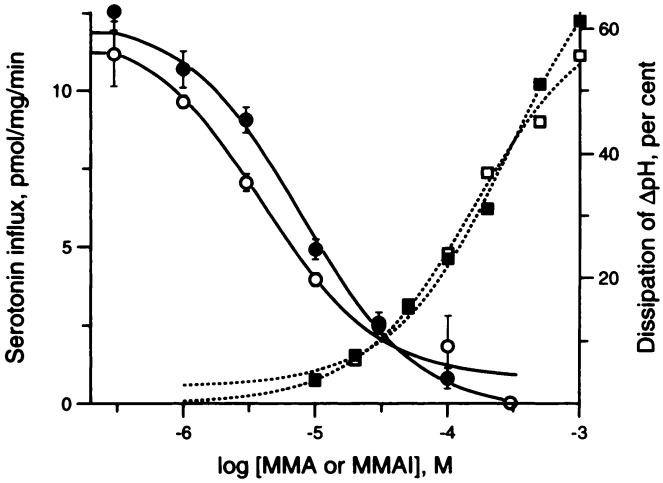

Fig. 3. Inhibition of serotonin transport and dissipation of ΔpH in chromaffin granule membrane vesicles by MMA and MMAI. For transport measurements (*circles*), MMA (*closed symbols*) or MMAI (*open symbols*) was added at the indicated concentration to the serotonin transport reaction, performed as described in Experimental Procedures. The control rate of transport was 28 pmol/mg/min in the absence of additions. The serotonin concentration used, 0.1 μM, is below the K_m (4.3 μM) (29). Half-maximal inhibition of transport required 7.7 ± 1.0 μM MMA or 3.9 ± 0.63 μM MMAI. For ΔpH dissipation measurements (*squares*), reversal of acridine orange quenching was determined at various MMA and MMAI concentrations as described in Experimental Procedures. The dissipation of ΔpH is presented relative to the effect of 20 mM NH_4Cl. Half-maximal dissipation of ΔpH required approximately 0.29 ± 0.05 mM MMA and 0.16 ± 0.3 mM MMAI. Each *point* represents the average of triplicate determinations for the transport measurements and duplicates for the ΔpH measurements. *Error bars* represent the standard deviation and are plotted only where they exceeded the size of the symbol.

amine transporter are 8.7-fold higher in the case of MMAI and 37-fold higher in the case of MMA, relative to the concentrations required to inhibit the plasma membrane serotonin transporter (compare with Fig. 1). Moreover, the order of potency is different; MMA is more potent at the plasma membrane, whereas MMAI is more potent at the vesicular membrane.

In synaptic vesicles, as well as chromaffin granules, the vacuolar ATPase pumps H^+ ions into the vesicle lumen, generating a transmembrane pH difference (ΔpH) that drives biogenic amine accumulation into these vesicles (24). Inhibition of serotonin accumulation could result from direct inhibition of the transporter, inhibition of the ATPase, or dissipation of ΔpH. In the experiment shown in Fig. 3, we tested the ability of MMA and MMAI to dissipate ΔpH or to inhibit its generation. In this experiment, ΔpH was measured by the quenching of acridine orange fluorescence. Vesicles incubated with Mg-ATP quench the fluorescence of acridine orange as they acidify (25), and agents that dissipate the ΔpH increase fluorescence toward the starting value. Fig. 3 shows that addition of increasing concentrations of MMA or MMAI to chromaffin granule membrane vesicles in the presence of Mg-ATP progressively increases internal pH. The increase in pH is probably due to the ability of MMA and MMAI, as weakly basic amines, to cross the vesicular membrane in the neutral form and bind internal H^+ ions. It is not likely to account for inhibition of serotonin accumulation by chromaffin granule membrane vesicles, which occurs at much lower concentrations (Fig. 3).

At concentrations of MMA and MMAI higher than those required for inhibition of serotonin transport into chromaffin granule membrane vesicles, both agents cause efflux of [^{3}H] serotonin from preloaded vesicles (Fig. 2). Because this efflux occurs at concentrations close to those required to dissipate ΔpH (see Fig. 3), it is likely that efflux represents a consequence of ΔpH dissipation, rather than a direct effect on the transporter.

Because the interactions of MMAI with the serotonin transporter were so similar to those of MDMA, we evaluated the specificity of MMAI by testing its ability to bind to the dopamine transporter and stimulate dopamine efflux. Fig. 4 shows the results of an experiment measuring the ability of MMAI, MDMA, and methamphetamine to displace the iodinated cocaine analog β-[^{125}I]CIT from platelet plasma membranes (Fig. 4A) and rat striatal membranes (Fig. 4B). β-[^{125}I]CIT binds with high affinity and specificity to both serotonin and dopamine transporters (26). It is apparent from the results in Fig. 4 that the two transporters have different specificities for amphetamine derivatives. Methamphetamine, which is the most potent inhibitor of β-CIT binding to the dopamine transporter, has little effect, at these concentrations, on β-CIT binding to the serotonin transporter. In contrast, MMAI is as effective as MDMA at inhibiting β-CIT binding to the serotonin transporter but is unable to displace β-CIT from the dopamine transporter.

The apparent lack of MMAI affinity for the dopamine transporter was confirmed by examining the ability of amphetamine derivatives to stimulate dopamine efflux from CV-1 cells stably transfected with cDNA encoding the rat brain dopamine transporter (Table 2). These cells were allowed to accumulate [^{3}H] dopamine and then 0.3 μM MDMA, MMAI, or methamphetamine was added. After an additional 2-min incubation, cells incubated with MMAI retained essentially all of the [^{3}H]dopamine that had accumulated internally, whereas cells incubated with MDMA or methamphetamine lost most of their accumulated radiolabel.

Discussion

The data presented here indicate that MMA and MMAI, although they are not neurotoxic toward serotonergic neurons (9, 10), display all of the *in vitro* characteristics of serotonin-releasing amphetamines such as MDMA and PCA (8, 11). The ability of these agents to inhibit serotonin accumulation and imipramine binding by platelet plasma membranes indicates that they interact directly with the serotonin transporter. Merely binding to the transporter, however, does not explain the ability of drugs like MDMA and PCA to stimulate serotonin efflux *in vivo* or *in vitro*. To account for efflux, we previously postulated that MDMA (8) and PCA (11) were substrates for the transporter and could exchange with internal serotonin. We reasoned that compounds that induce efflux from [^{3}H] serotonin-loaded platelet plasma membrane vesicles are likely to be substrates if the efflux is inhibited by imipramine and requires Na^+. By these criteria, both MMA and MMAI are substrates for the serotonin transporter and induce serotonin efflux by transporter-mediated exchange. Both drugs are more potent than MDMA at inhibiting transport and binding and stimulating efflux and would be expected to cause serotonin release from serotonergic terminals *in vivo*.

The lack of neurotoxicity associated with administration of MMA and MMAI strongly suggests that inducing serotonin release is not sufficient to cause the destruction of serotonergic terminals. It is, however, likely to be a contributing factor.

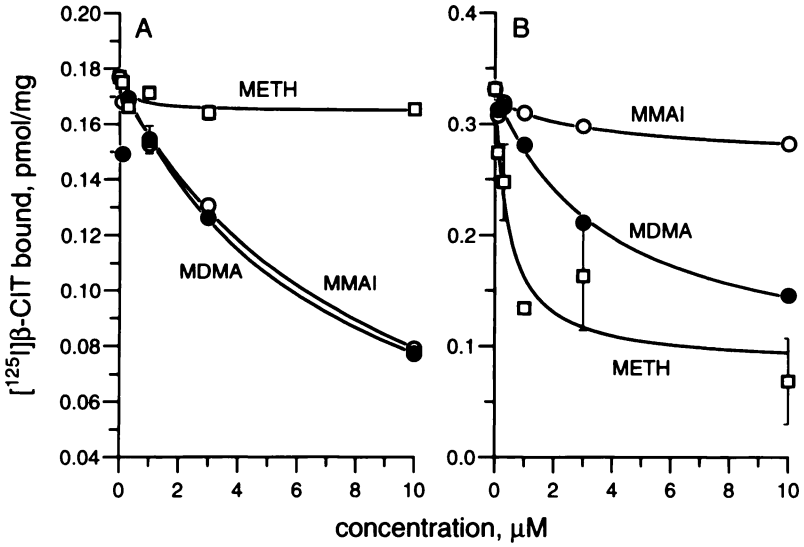

Fig. 4. Inhibition of β-CIT binding to serotonin transporters in platelet plasma membranes (A) and to dopamine transporters in rat striatal membranes (B). Approximately 50–70 μg of platelet plasma membrane vesicle protein at a concentration of 8–10 mg/ml or 15–20 μg of striatal membrane protein at a concentration of 1.5 mg/ml were diluted with 150 mM Na_2SO_4, 10 mM lithium borate, pH 9.5, 1 mM $MgSO_4$, to a final volume of 300 μl containing 0.05 nM β-[^{125}I]CIT and were incubated at 25° for 30 min in the presence of the indicated concentration of MMAI, MDMA, or methamphetamine (*METH*). Citalopram (5 μM) was present in all incubations containing striatal membranes to prevent binding to serotonin transporters. After this incubation, the reaction was terminated by dilution and filtration and the samples were counted as described for imipramine in Experimental Procedures. Each *point* represents the mean of triplicate measurements. Standard deviations are shown as *error bars* where they exceed the size of the symbol.

TABLE 2

Dopamine efflux from dopamine transporter cDNA-transfected CV-1 cells

CV1-DAT cells were obtained from Dr. Beth Hoffman, Laboratory of Cell Biology, National Institute of Mental Health. These cells have been stably transfected with cDNA encoding a rat brain dopamine transporter inserted in the pRC/CMV vector (Invitrogen, San Diego, CA), using the $CaPO_4$ procedure (28). The CV1-DAT line was selected by G418 resistance (conferred by pRC/CMV) and the ability to accumulate dopamine to high intracellular levels. Monolayers grown in 24-well plates, in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 400 mg/liter G418, were washed with phosphate-buffered saline containing 0.1 mM $CaCl_2$ and were incubated for 10 min at room temperature with 200 μl/well of the same buffer containing 20 nM [^{3}H]dopamine. Other experiments demonstrated that dopamine accumulation had reached steady state at 5 min and remained constant for at least 20 min (data not shown). At 10 min after [^{3}H]dopamine addition, 0.3 μM MMAI, MDMA, or methamphetamine was added and the reaction was allowed to proceed for 2 min. The cells were then rinsed with phosphate-buffered saline, dissolved in 0.4 ml/well of 1% sodium dodecyl sulfate, and counted in 3 ml of Optifluor. Control uptake (no additions) was approximately 1.2 pmol/well.

Additions	[^{3}H]Dopamine remaining
	% of control
None	100.0 ± 9.6
MMAI	78.4 ± 7.04
MDMA	23.2 ± 2.4
Methamphetamine	24.9 ± 2.0

Johnson and Nichols (10) demonstrated that levels of amphetamine that had no effect on cortical levels of serotonin, 5-hydroxyindole acetic acid, or paroxetine-binding activity nevertheless decreased all three serotonergic parameters if amphetamine was coadministered with MMAI. At the doses used, MMAI had no effect by itself. They concluded that dopamine release plays a major role in the neurotoxicity of MDMA and related drugs that release both dopamine and serotonin. The lack of MMAI toxicity was suggested to result from its ability to specifically release serotonin without releasing dopamine. The results presented here support the proposal that MMAI and MMA release serotonin at least as well as does MDMA. Moreover, our results indicate that MMAI interacts with the serotonin transporter at concentrations that have little effect on the dopamine transporter.

The mechanisms by which serotonin is stored within synaptic vesicles represent another potential target for the action of neurotoxic amphetamines. MDMA (8) and PCA (11) both inhibit serotonin transport into chromaffin granule membrane vesicles and both, like amphetamine (27), dissipate the ΔpH. The results presented in Fig. 3 indicate that both MMA and MMAI inhibit serotonin accumulation by chromaffin granule membrane vesicles containing the vesicular biogenic amine transporter and both compounds also dissipate the ΔpH generated by ATP in these membranes, consequently inducing serotonin efflux. Both MMA and MMAI are at least as potent as MDMA for these activities. Although the effect on ΔpH is likely to result from nonionic diffusion of the weakly basic amines, inhibition of transport occurs at lower concentrations of MMA and MMAI and may be due to direct interaction with the transporter. Thus, all of the effects of MDMA on plasma membrane and vesicular serotonin transport systems are reproduced with MMA and MMAI. The difference in neurotoxicity between these agents is unlikely to reflect a difference in their ability to interact with serotonin transport systems. However, MDMA was much more effective than MMAI at inhibiting β-[^{125}I]CIT binding to the dopamine transporter and at stimulating dopamine efflux from cells expressing the dopamine transporter. Thus, the differences in neurotoxicity between the two compounds may reflect their transporter specificity rather than any qualitative difference in their mechanism of action.

References

1. Ricaurte, G., G. Bryan, L. Strauss, L. Seiden, and C. Schuster. Hallucinogenic amphetamine selectively destroys brain serotonin nerve terminals. *Science (Washington D. C.)* **229**:986–988 (1985).
2. Mamounas, L. A., and M. E. Molliver. Evidence for dual serotonergic projections to neocortex: axons from the dorsal and median raphe nuclei are differentially vulnerable to the neurotoxin *p*-chloroamphetamine (PCA). *Exp. Neurol.* **102**:23–36 (1988).
3. Molliver, D. C., and M. E. Molliver. Anatomic evidence for a neurotoxic effect of (±)-fenfluramine upon serotonergic projections in the rat. *Brain Res.* **511**:165–168 (1990).
4. O'Hearn, E., G. Battaglia, E. B. DeSouza, M. J. Kuhar, and M. E. Molliver. Methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) cause selective ablation of serotonergic axon terminals in forebrain: immunocytochemical evidence for neurotoxicity. *J. Neurosci.* **8**:2788–2803 (1988).
5. Fuller, R. W. Mechanism by which uptake inhibitors antagonize *p*-chloroampehtamine-induced depletion of brain serotonin. *Neurochem. Res.* **5**:241–245 (1980).
6. Stone, D. M., M. Johnson, G. R. Hanson, and J. B. Gibb. Role of endogenous dopamine in the central serotonergic deficits induced by 3,4-methylenedioxymethamphetamine. *J. Pharmacol. Exp. Ther.* **247**:79–87 (1988).
7. Schmidt, C. J., C. K. Black, and V. L. Taylor. Antagonism of the neurotoxicity

due to a single administration of methylenedioxymethamphetamine. *Eur. J. Pharmacol.* **181**:59–70 (1990).
8. Rudnick, G., and S. C. Wall. The molecular mechanism of ecstasy [3,4-methylenedioxymethamphetamine (MDMA)]: serotonin transporters are targets for MDMA-induced serotonin release. *Proc. Natl. Acad. Sci. USA* **89**:1817–1821 (1992).
9. Nichols, D. E., W. K. Brewster, M. P. Johnson, R. Oberlender, and R. M. Riggs. Nonneurotoxic tetralin and indan analogues of 3,4-(methylenedioxy)amphetamine (MDA). *J. Med. Chem.* **33**:703–710 (1990).
10. Johnson, M. P., and D. E. Nichols. Combined administration of a nonneurotoxic 3,4-methylenedioxymethamphetamine analogue with amphetamine produces serotonin neurotoxicity in rats. *Neuropharmacology* **30**:819–822 (1991).
11. Rudnick, G., and S. C. Wall. *p*-Chloroamphetamine induces serotonin release through serotonin transporters. *Biochemistry* **31**:6710–6718 (1992).
12. Blakely, R., H. Berson, R. Fremeau, M. Caron, M. Peek, H. Prince, and C. Bradely. Cloning and expression of a functional serotonin transporter from rat brain. *Nature (Lond.)* **354**:66–70 (1991).
13. Hoffman, B. J., E. Mezey, and M. J. Brownstein. Cloning of a serotonin transporter affected by antidepressants. *Science (Washington D. C.)* **254**:579–580 (1991).
14. Njus, D., P. M. Kelley, and G. J. Harnadek. Bioenergetics of secretory vesicles. *Biochim. Biophys. Acta* **853**:237–265 (1986).
15. Neumeyer, J. L., S. Wang, R. A. Milius, R. M. Baldwin, Y. Zea-Ponce, P. B. Hoffer, E. Sybirska, M. Al-Tikriti, D. Charney, R. T. Malison, M. Laruelle, and R. B. Innis. [^{123}I]-2β-Carbomethoxy-3β-(4-iodophenyl)tropane: high-affinity SPECT radiotracer of monoamine reuptake sites in brain. *J. Med. Chem.* **34**:3144–3146 (1991).
16. Barber, A. J., and G. A. Jamieson. Isolation and characterization of plasma membranes from human blood platelets. *J. Biol. Chem.* **245**:6357–6365 (1970).
17. Rudnick, G., and P. Nelson. Platelet 5-hydroxytryptamine transport: an electroneutral mechanism coupled to potassium. *Biochemistry* **17**:4739–4742 (1978).
18. Schuldiner, S., H. Fishkes, and B. I. Kanner. Role of a transmembrane pH gradient in epinephrine transport by chromaffin granule membrane vesicles. *Proc. Natl. Acad. Sci. USA* **75**:3713–3716 (1978).
19. Humphreys, C. J., J. Levin, and G. Rudnick. Antidepressant binding to the porcine and human platelet serotonin transporters. *Mol. Pharmacol.* **33**:657–663 (1988).
20. Rudnick, G. Active transport of 5-hydroxytryptamine by plasma membrane vesicles isolated from human blood platelets. *J. Biol. Chem.* **252**:2170–2174 (1977).
21. Rudnick, G. Serotonin transport in plasma and dense granule membrane vesicles, in *Platelet Responses and Metabolism* (H. Holmsen, ed.). CRC Press, Boca Raton, FL, 119–133 (1986).
22. Talvenheimo, J., P. J. Nelson, and G. Rudnick. Mechanism of imipramine inhibition of platelet 5-hydroxytryptamine transport. *J. Biol. Chem.* **254**:4631–4635 (1979).
23. Nelson, P., and G. Rudnick. Coupling between platelet 5-hydroxytryptamine and potassium transport. *J. Biol. Chem.* **254**:10084–10089 (1979).
24. Rudnick, G. ATP-driven H^+ pumping into intracellular organelles. *Annu. Rev. Physiol.* **48**:403–413 (1986).
25. Rottenberg, H. The measurement of membrane potential and increased pH in cells, organelles, and vesicles. *Methods Enzymol.* **55**:547–569 (1979).
26. Wall, S. C., R. B. Innis, and G. Rudnick. Binding of the cocaine analog 2β-carbomethoxy-3β-(4- [^{125}I]iodophenyl)tropane to serotonin and dopamine transporters: different ionic requirements for substrate and 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl)tropane binding. *Mol. Pharmacol.* **43**:264–270 (1993).
27. Sulzer, D., and S. Rayport. Amphetamine and other psychostimulants reduce pH gradient in midbrain dopaminergic neurons and chromaffin granules: a mechanism of action. *Neuron* **5**:797–808 (1990).
28. Sambrook, J., E. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
29. Kanner, B. I., H. Fishkes, R. Maron, I. Sharon, and S. Schuldiner. Reserpine as a competitive and reversible inhibitor of the catecholamine transporter of bovine chromaffin granules. *FEBS Lett.* **100**:175–178 (1979).

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