

Amphetamine Derivatives Interact with Both Plasma Membrane and Secretory Vesicle Biogenic Amine Transporters

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SUMMARY

The interaction of fenfluramine, 3,4-methylenedioxymethamphetamine (MDMA), and *p*-chloroamphetamine (PCA) with the platelet plasma membrane serotonin transporter and the vesicular amine transporter were studied using both transport and binding measurements. Fenfluramine is apparently a substrate for the plasma membrane transporter, and consequently inhibits both serotonin transport and imipramine binding. Moreover, fenfluramine exchanges with internal [³H]serotonin in a plasma membrane transporter-mediated reaction that requires NaCl and is blocked by imipramine. These properties are similar to those of MDMA and PCA as previously described. In adrenal chromaffin granule membrane vesicles containing the vesicular amine trans-

porter, fenfluramine inhibited serotonin transport and dissipated the transmembrane pH difference (Δ pH) that drives amine uptake. The use of [³H]reserpine-binding measurements to determine drug interaction with the vesicular amine transporter allowed assessment of the relative ability of MDMA, PCA, and fenfluramine to block the substrate site of the vesicular transporter. These measurements permit a distinction between inhibition of vesicular serotonin transport by directly blocking vesicular amine transport and by dissipating Δ pH. The results indicate that MDMA and fenfluramine inhibit by both mechanisms but PCA dissipates Δ pH without blocking vesicular amine transport directly.

Amphetamine and its derivatives are well-known as sympathomimetic amines that act indirectly by releasing endogenous biogenic amines (1). This release has been attributed to the ability of extracellular amphetamines to exchange with cytoplasmic biogenic amines (2, 3). Exchange was proposed to be catalyzed by the transporters that normally serve to inactivate transmitter action by reuptake of norepinephrine, dopamine, and serotonin. An additional mechanism has been proposed in which biogenic amine efflux is stimulated through dissipation of the transmembrane Δ pH that drives biogenic amine uptake into synaptic vesicles (4, 5).

A number of amphetamine derivatives preferentially release serotonin and also cause the degeneration of serotonergic nerve endings. These include the designer drug MDMA (also known as "ecstasy"), PCA, and the appetite suppressant fenfluramine. Serotonin release by these agents does not require Ca²⁺, and is inhibited by serotonin transport blockers, leading to the proposal that these agents exchange with intracellular serotonin through the action of the plasma membrane serotonin transporter (6, 7). We have demonstrated *in vitro* exchange of serotonin for MDMA and PCA using platelet plasma mem-

brane vesicles containing the serotonin transporter (8, 9). In the present study, we extend these results to fenfluramine, which demonstrates a similar ability to serve as a substrate for the serotonin transporter.

The extent of serotonin depletion by MDMA, PCA, and fenfluramine indicates that serotonin stored in synaptic vesicles, as well as cytoplasmic serotonin, is mobilized by these agents. Serotonin is accumulated within synaptic vesicles by a vesicular amine transporter that utilizes the Δ pH generated by the vacuolar ATPase (10). Previous studies with amphetamine derivatives have demonstrated their ability to dissipate this Δ pH by nonionic diffusion and to block serotonin transport (4, 5, 8, 9). As amines, however, the amphetamine derivatives are potential substrates and inhibitors of the vesicular amine transporter. [³H]Reserpine binding to chromaffin granule membranes is blocked by substrates for the transporter and has been used as an indicator of its substrate binding site. In the present study, we demonstrate that MDMA and fenfluramine, but not PCA, inhibit [³H]reserpine binding to the vesicular amine transporter. These results point out differences between serotonin-releasing amphetamine derivatives that may be important to their pharmacologic and behavioral effects.

Experimental Procedures

Materials. [³H]serotonin (12.6 Ci/mmol) and [³H]imipramine (40.4 Ci/mmol) were purchased from DuPont-New England Nuclear (Boe-

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ton, MA). [^3H]Reserpine (20 Ci/mmol) was purchased from the Nuclear Research Centre (Negev, Israel). All other reagents were reagent grade, purchased from commercial sources.

Preparation of membranes. Outdated human platelet concentrates were purchased from the Connecticut Red Cross, Farmington, CT. Platelets from 50 to 100 individuals were pooled for each membrane preparation. Platelet plasma membrane vesicles were isolated by the method of Barber and Jamieson (11) with the modifications described previously (12). Chromaffin granule membrane vesicles were prepared as described by Schuldiner et al. (13) by repeated osmotic lysis of bovine adrenal medullary chromaffin granules isolated by differential sedimentation.

Binding assays. Imipramine binding was measured at 25° using the filtration assay described previously (14). 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 [^3H]imipramine (19–23 cpm/fmol). After a 20-min incubation, the reactions (300 μl per assay) were terminated by dilution with 4-ml, ice-cold, iso-osmotic NaCl and filtered through Whatman GF/B filters 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 less than 5% of the mean.

Measurements of reserpine binding were performed as described (15). Membranes were diluted to a protein concentration of approximately 0.15 mg/ml in a solution containing 0.3 M sucrose, 10 mM K-HEPES (pH 8.5), 2.5 mM MgSO_4 , 5 mM KCl, 5 mM ATP (where indicated), and 1 nM [^3H]reserpine. The mixture was incubated at 37° and after 30 min (4 hr in samples where ATP was omitted), a 200- μl sample of the suspension was applied to a 3-ml column of Sephadex G-50 (Pharmacia; Uppsala, Sweden). The column was prepacked in a disposable syringe by centrifugation for 90 seconds in a clinical centrifuge at $100 \times g$. After sample application, the column was centrifuged for 2 min at $200 \times g$ and the effluent was assayed for radioactivity.

Transport assays. Transport of [^3H]serotonin into plasma membrane vesicles was measured at 25° using the previously described filtration assay (16). Unless otherwise indicated, vesicles were equilibrated with a 10 mM lithium phosphate buffer (pH 6.7) containing 133 mM K_2SO_4 , and 1 mM MgSO_4 , and diluted into an external medium of 0.2 M NaCl containing 10 mM lithium phosphate buffer (pH 6.7), 1 mM MgSO_4 , and 0.1 μM [^3H]serotonin. Transport into chromaffin granule membrane vesicles was measured at 37°. The vesicles were diluted to a concentration of approximately 0.25 mg/ml in 0.3 M sucrose containing 10 mM K-HEPES (pH 8.5), 5 mM KCl, 2.5 mM MgSO_4 , 5 mM disodium ATP, and 0.4 μM [^3H]serotonin (unless otherwise indicated). Reactions (200 μl per assay) were stopped by dilution, filtration, and washing, and filtered vesicles were counted as described previously (13). The standard error of replicate assay values was typically less than 5% of the mean.

Exchange. Platelet plasma membrane vesicles equilibrated with a 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 [^3H]serotonin at 25°. After serotonin accumulation had reached a maximum (5–10 min), efflux was initiated by diluting the suspension 40-fold with the indicated medium. Thirty seconds after dilution, the vesicles were collected by filtering 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. 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 K-HEPES (pH 8.5), 5 mM KCl, 2.5 mM MgSO_4 , 5 mM disodium ATP, and 0.1 μM [^3H]serotonin, and efflux was measured after dilution into the same medium without ATP, MgSO_4 , or [^3H]

serotonin. The standard error of replicate assay values was typically less than 10% of the mean.

ΔpH measurements. Chromaffin granule membrane vesicles (80 μg protein) were incubated at room temperature in a cuvette containing 2 ml of 10 mM Tris-*N*-(2-hydroxyethyl)piperazine-*N*-3-propanesulfonic acid (pH 8.5), 150 mM KCl, 6 μM acridine orange, and 5 mM ATP. The relative fluorescence of the mixture was measured using an excitation wavelength of 490 nm and an emission wavelength of 526 nm on a Perkin-Elmer LS-5B Luminescence Spectrometer. When the baseline 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 fenfluramine 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 fenfluramine required for half-maximal reversal of the fluorescence quenching varied by less than 10% of the mean value.

Results

Fenfluramine interaction with the plasma membrane serotonin transporter. Previous results with MDMA (9) and PCA (8) indicated that these amphetamine derivatives interacted directly with the serotonin transporter of platelet plasma membrane as demonstrated by their ability to compete with serotonin for transport and with imipramine for binding. Furthermore, these compounds exchange with serotonin in a transporter-mediated process and are likely to be substrates for the transporter. To test the generality of this process, we examined the ability of another amphetamine derivative, fenfluramine (*N*-ethyl- α -methyl-*m*-(trifluoromethyl)phenethylamine), to interact with the serotonin transporter.

Fig. 1 (left panel) presents data indicating that fenfluramine is a competitive inhibitor of serotonin transport. Initial rates of [^3H]serotonin influx into platelet plasma membrane vesicles were measured in the presence and absence of 0.5 μM fenfluramine over a range of [^3H]serotonin concentrations. In this Eadie-Hofstee plot (17), the ordinate intercept is unchanged in the presence of fenfluramine, but the K_m (–slope) is increased. This behavior is expected both for a competitive inhibitor of serotonin transport and for a substrate of the transporter. From the increase in K_m , a K_i of 0.59 μM was calculated for fenfluramine.

In the experiment shown in the center panel of Fig. 1 as a Scatchard plot (18), the ability of fenfluramine to inhibit [^3H]imipramine binding to the transporter was measured over a range of [^3H]imipramine concentrations. In this plot, the *abscissa intercept* represents the total number of binding sites and is unchanged by addition of 10 μM fenfluramine. The apparent K_d for imipramine is increased by fenfluramine, as demonstrated by the change in the slope ($-K_d^{-1}$). From the magnitude of the increase, we calculated a K_d of 4.1 μM for fenfluramine. Although this result demonstrates a direct interaction between fenfluramine and the transporter binding site, it does not distinguish between a substrate and a competitive transport inhibitor.

The ability to stimulate [^3H]serotonin efflux from membrane vesicles is common to transporter substrates such as serotonin, MDMA, and PCA (8, 9, 19). In the experiment shown in the right panel of Fig. 1, vesicles preloaded with NaCl and [^3H]serotonin were diluted into a [^3H]serotonin-free medium containing the indicated concentrations of fenfluramine. In the

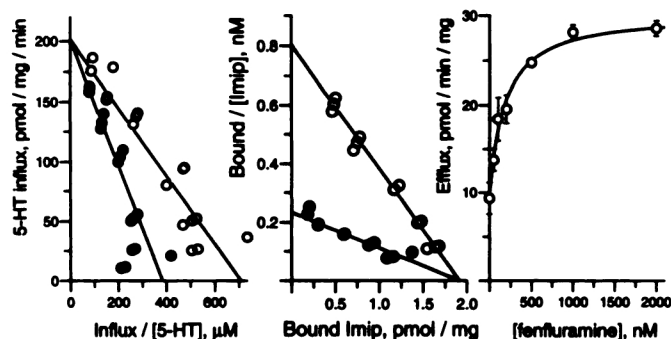


Fig. 1. Interaction of fenfluramine with the plasma membrane serotonin transporter. *Left panel*, competitive inhibition of serotonin influx. Initial rates of [^3H]serotonin transport were measured as described in Experimental Procedures over a range of serotonin concentrations in the presence (●) and absence (○) of 0.5 μM fenfluramine. Nonspecific influx (measured in the absence of Na^+) was subtracted to give the net rates. Data are plotted according to the method of Hofstee (17). From the K_m for serotonin in the presence (0.53 μM) and absence (0.29 μM) of fenfluramine, the K_i for fenfluramine was calculated to be 0.59 μM . *Center panel*, competitive inhibition of [^3H]imipramine binding. Equilibrium imipramine binding was measured as described in Experimental Procedures over a range of imipramine concentrations in the presence (●) and absence (○) of 10 μM fenfluramine. Nonspecific binding (measured in the presence of 100 μM cocaine) was subtracted to give net binding. Data are plotted according to the method of Scatchard (18). From the K_d for imipramine in the presence (8.2 nM) and absence (2.4 nM) of fenfluramine, the K_i for fenfluramine was calculated to be 4.1 μM . *Right panel*, stimulation of serotonin efflux. Vesicles preloaded with [^3H]serotonin were diluted 40-fold into a [^3H]serotonin-free medium containing the indicated concentrations of fenfluramine. After 30 seconds, the amount of [^3H]serotonin remaining in the vesicles was measured and efflux was calculated relative to the amount present before dilution. Half-maximal stimulation of efflux occurred at 170 nM fenfluramine. Each of the panels of this figure shows results from a representative experiment. Each experiment was repeated 3 to 4 times yielding K_i , K_d , and half-maximal efflux stimulation values that varied from 10 to 30% between experiments.

absence of external K^+ , efflux under these conditions is stimulated by exchange of internal [^3H]serotonin with an external substrate (8, 9, 19). As shown in Fig. 1, fenfluramine stimulates [^3H]serotonin efflux under these conditions with half-maximal stimulation evident at 170 nM fenfluramine. Although not shown, fenfluramine-stimulated efflux was dependent on external NaCl and was blocked by 1 μM imipramine, demonstrating that efflux represented transporter-mediated exchange and not a nonspecific effect of fenfluramine.

Effect of fenfluramine on serotonin transport into secretory vesicles. In membrane vesicles isolated from bovine adrenal chromaffin granules, as in synaptic vesicles, serotonin accumulation is driven by an electrochemical H^+ potential (10). MDMA and PCA inhibit [^3H]serotonin accumulation by chromaffin granule membrane vesicles and also dissipate the ΔpH generated by ATP-dependent H^+ pumping in these vesicles (8, 9). The data in Fig. 2 show that fenfluramine inhibits ATP-dependent [^3H]serotonin transport into chromaffin granule membrane vesicles with a $K_{0.5}$ of 2.2 μM , and at higher concentrations, dissipates the ΔpH generated by ATP-dependent H^+ pumping, as measured by reversal of acridine orange quenching. In vesicles that have accumulated [^3H]serotonin, dissipation of the ΔpH is known to cause efflux of radiolabel (20). Fig. 2 shows that efflux of [^3H]serotonin is also observed at concentrations of fenfluramine that significantly dissipate ΔpH ($K_{0.5}$ = 168 μM).

Amphetamine derivative binding to the reserpine site

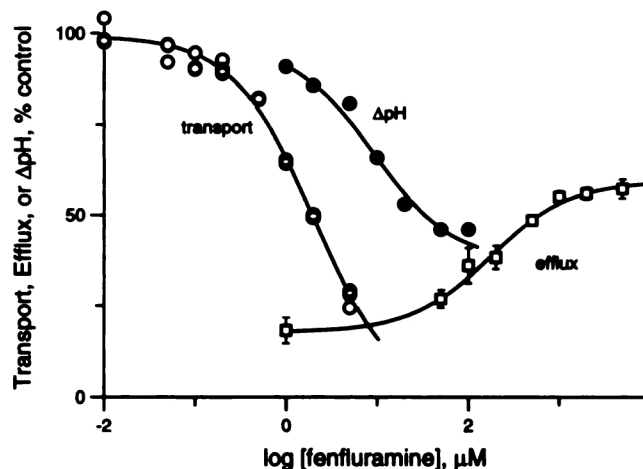


Fig. 2. Effects of fenfluramine on chromaffin granule membrane vesicles. The ability of indicated concentrations of fenfluramine to inhibit serotonin transport (○), dissipate ΔpH (●), and stimulate serotonin efflux (□) was determined as described in Experimental Procedures. The concentration of fenfluramine needed for half-maximal effect was 2.2 μM for transport inhibition, 9 μM for dissipation of ΔpH , and 168 μM for efflux stimulation.

of the vesicular amine transporter. Fenfluramine and other amphetamine derivatives such as MDMA and PCA could potentially inhibit ATP-dependent [^3H]serotonin transport by two mechanisms. Dissipation of ΔpH by nonionic diffusion of amphetamine derivatives removes the driving force for transport. Because influx of each amine molecule requires efflux of two H^+ ions (21, 22), transport is particularly sensitive to small changes in intravesicular pH. Alternatively, amphetamine derivatives can potentially interact directly with the substrate-binding site to competitively inhibit transport as previously suggested for PCA (8). Because substrates for the vesicular amine transporter are known to compete with [^3H]reserpine for binding (23), we measured the ability of fenfluramine, MDMA, and PCA to inhibit binding of [^3H]reserpine to chromaffin granule membranes.

Fig. 3 shows the results of experiments in which we measured the inhibition by fenfluramine, MDMA, and PCA of serotonin transport and reserpine binding in chromaffin granule membrane vesicles. Reserpine binding is accelerated by the electrochemical H^+ potential (ΔH^+) generated by ATP-driven H^+ pumping (15, 23, 24). Thus, inhibition of [^3H]reserpine binding could result from competition at the reserpine-binding site or dissipation of ΔpH . By measuring [^3H]reserpine binding in the presence and absence of ATP, we were able to distinguish between these two mechanisms. [^3H]serotonin transport was measured under standard reaction conditions in the presence of ATP (13). The results in Fig. 3 show that in all cases, inhibition of transport occurred at lower concentrations of the amphetamine derivative than did inhibition of [^3H]reserpine binding. Half-maximal transport inhibition required 2.0 μM fenfluramine, 2.7 μM MDMA, and 1.5 μM PCA.

Half-maximal inhibition of [^3H]reserpine binding occurred at 30 μM fenfluramine, whether ATP-dependent or -independent binding was measured (Fig. 3A). Similar results were obtained with MDMA (Fig. 3B) ($K_{0.5}$ = 235 μM). In contrast, PCA inhibited ATP-dependent [^3H]reserpine binding but had practically no effect on binding measured in the absence of ATP (Fig. 3C). Half-maximal inhibition of ATP-dependent [^3H]reserpine binding required 119 μM PCA, but concentrations of

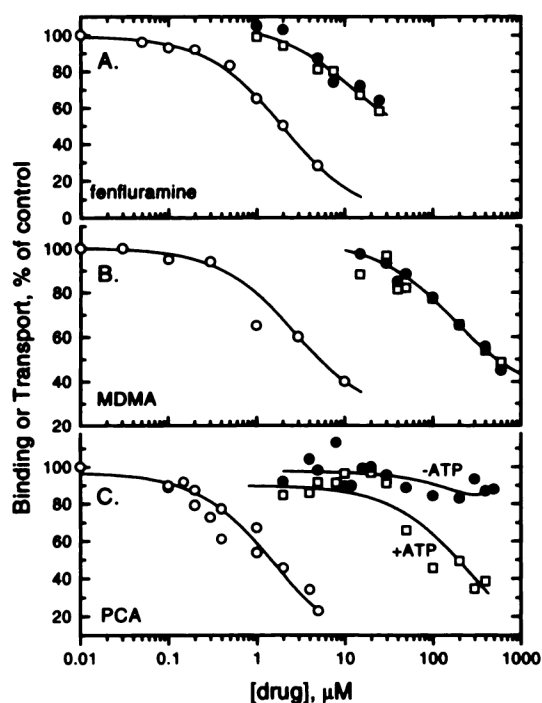


Fig. 3. Inhibition of chromaffin granule [^3H]serotonin transport and [^3H]reserpine binding by amphetamine derivatives. The ability of fenfluramine, MDMA, and PCA to inhibit [^3H]serotonin influx (○) and reserpine binding was measured as described in Experimental Procedures. Reserpine binding was measured in the presence (□) and absence (●) of ATP. Half-maximal inhibition of transport occurred at 1.96, 2.7, and 1.53 μM for fenfluramine, MDMA, and PCA, respectively. Half-maximal inhibition of ATP-dependent reserpine binding required 30, 235, and 119 μM , respectively, of fenfluramine, MDMA, and PCA.

PCA as high as 800 μM failed to inhibit more than 10% of nonenergized [^3H]reserpine binding. This observation indicates that PCA inhibits predominantly by dissipating the ATP-generated $\Delta\mu_{\text{H}^+}$ that accelerates reserpine binding.

Apparent competitive inhibition by weak bases. We had previously concluded that PCA interacts directly with the vesicular amine transporter (8). This conclusion was based on results showing apparent competitive inhibition with serotonin for transport into chromaffin granule membrane vesicles. Because the results in Fig. 3C indicate that PCA does not interact directly with the substrate site of the vesicular amine transporter, we tested the ability of other weak bases to mimic competitive inhibition of serotonin transport. Results from independent experiments (not shown) show that ammonium chloride, which dissipates ΔpH by nonionic diffusion, inhibits the initial rate of serotonin transport and that the inhibition resembles that of a competitive substrate with a K_i of approximately 450 μM . The competitive nature of the inhibition apparently results from an increase in the K_m for substrate as the ΔpH , which drives transport, is decreased.

Discussion

The ability of amphetamine derivatives to release biogenic amines from nerve endings is likely to result from interference with biogenic amine transport systems at both the plasma membrane and the synaptic vesicle. The results presented here with fenfluramine extend our previous results using MDMA (9), PCA (8), 3-methoxy-4-methylamphetamine, and 5-methoxy-6-methyl-2-aminindan (25). In all cases tested, amphet-

amine derivatives that release serotonin compete with substrate and ligands for sites on the plasma membrane serotonin transporter and also inhibit serotonin accumulation by chromaffin granule membrane vesicles.

Previously, our understanding of amphetamine interaction with the vesicular transport system was limited to measurements of transport and ΔpH . In the studies presented here, we have also evaluated the ability of amphetamine derivatives to interact directly with the vesicular amine transporter by measuring their effect on [^3H]reserpine binding. Reserpine is thought to bind directly to the substrate site of the transporter and is, therefore, a more precise indicator of interaction with that site (23). From the present studies, it is clear that fenfluramine and MDMA inhibit vesicular amine transport by two mechanisms: dissipation of ΔpH and competition for the substrate binding site. However, PCA does not compete for the substrate site but only dissipates ΔpH .

Inhibition of vesicular amine transport by fenfluramine, MDMA, and PCA occurs at concentrations below those required to dissipate the ATP-generated ΔpH as measured by reversal of acridine orange quenching (8, 9) (Fig. 2). Amphetamine derivatives can potentially inhibit transport by a number of mechanisms, including direct competition for the transporter substrate site and dissipation of the ΔpH that drives transport. The ability of these compounds to dissipate ΔpH at low concentrations indicates that they act as protonophores. The observation that transport inhibition occurs at lower concentrations could result from high affinity binding of amphetamine derivatives to the transporter at concentrations too low to affect ΔpH . However, we consider it more likely that a decrease in ΔpH is responsible for both effects and that the differential inhibition is a result of the higher sensitivity of transport to the uncoupling effects of amphetamines. It is well established that two H^+ ions are formally exchanged for each biogenic amine molecule transported (21, 22). This coupling, together with the known electrogenicity of the transport process (26, 27) dictates that the proton conducting activity of amphetamine derivatives will affect transport more profoundly than acridine orange quenching measurements would predict.

If interaction with the transport site were the only mechanism for amphetamine inhibition of transport, then binding of [^3H]reserpine to that site would show the same sensitivity as transport. The results in Fig. 3 clearly demonstrate that inhibition of reserpine binding requires concentrations of the amphetamine derivatives higher than those that block transport. Transport inhibition occurs at roughly the same concentration for fenfluramine, MDMA, and PCA (Fig. 3). Inhibition of [^3H]reserpine binding, by comparison, occurs at different concentrations for the three compounds. In the absence of ATP, where ΔpH dissipation is not a factor, binding is half-maximal at approximately 20 μM fenfluramine, 500 μM MDMA, and not detected for PCA (●, Fig. 3). We previously demonstrated that [^3H]reserpine binding is less sensitive than transport to agents that decrease ΔpH (15). Moreover, transport inhibitors that do not dissipate ΔpH , such as ethidium, block transport and binding at similar concentrations.¹ It is likely, therefore, that the increased sensitivity of amine transport to amphetamine

¹ R. Yelin, S. Steiner-Mordoch, and S. Schuldiner, The vesicular monoamine transporter is a novel type of multidrug transporter. Submitted for publication.

derivatives results from the proton-conducting actions of these agents.

Efflux of accumulated [^3H]serotonin induced by fenfluramine, MDMA, PCA, 3-methoxy-4-methylamphetamine, and 5-methoxy-6-methyl-2-aminoindan also occurs only at concentrations above those required to inhibit transport (Fig. 2) (8, 9, 25). Multiple mechanisms, including increased intravesicular pH, have previously been proposed to explain the difference in amphetamine concentrations required to inhibit uptake and stimulate efflux of norepinephrine from vas deferens (28). Efflux of accumulated [^3H]serotonin from chromaffin granule membrane vesicles is known to require significant dissipation of ΔpH (20). Thus, the higher potency of amphetamine derivatives for inhibiting vesicular transport relative to stimulating efflux is again likely to reflect the different sensitivity of the two processes to changes in ΔpH .

In the case of the the plasma membrane serotonin transporter, higher concentrations of amphetamine derivatives are required to inhibit imipramine binding relative to transport (Fig. 1). This effect is likely to result from the different assays used to evaluate binding and transport. For substrates, like amphetamine derivatives, the K_i for transport inhibition is the same as the K_m for transport, although the K_i for inhibition of binding reflects the K_d of the inhibitor. Substrates for these transporters frequently have K_m values lower than the K_d for binding (29, 30). The higher sensitivity of vesicular transport relative to reserpine binding may also partially reflect the influence of transport kinetics on the apparent potency of the inhibitors.

It may be significant that of the compounds tested, only PCA apparently did not bind to the reserpine site of the vesicular amine transporter, as judged by its inability to block [^3H]reserpine binding in the absence of ATP (Fig. 3C). PCA is the only one of these compounds with a halogen as a ring substituent. Halogenated aromatic rings frequently display aberrant behavior in structure-activity relationships because the halogen can both release and withdraw electrons (31). The best known substrates for the vesicular amine transporter have ring substituents (such as $-\text{OH}$ or $-\text{CH}_3$) that are strong electron-releasing substituents. The absence of an N -alkyl group in PCA is not likely to explain the lack of affinity because 3,4-methylenedioxymphetamine, which is also a primary amine, inhibits [^3H]reserpine binding in the absence of ATP (data not shown).

Despite the apparent differences in mechanism, all the compounds tested inhibited both the plasma membrane and vesicular transport systems. All of these compounds are known to be neurotoxic toward serotonin terminals, but their behavioral effects are different. Whether the lack of PCA binding to the reserpine site of the vesicular amine transporter is responsible for its different behavioral properties remains an unanswered question.

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