

Biogenic Amine Flux Mediated by Cloned Transporters Stably Expressed in Cultured Cell Lines: Amphetamine Specificity for Inhibition and Efflux

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SUMMARY

LLC-PK₁ cells have been stably transfected with cDNAs encoding the human norepinephrine transporter (NET), rat dopamine transporter (DAT), and rat serotonin transporter. Using these cell lines, the specificity of each transporter toward agents that inhibit substrate influx and stimulate substrate efflux across the plasma membrane was examined. With 1-methyl-4-phenylpyridinium as a substrate for DAT and NET and serotonin as a substrate for the serotonin transporter, each transporter demonstrated a distinct pattern of inhibition by a panel of amphetamine derivatives and analogs, including amphetamine, methamphetamine (also known as "ecstasy"), *p*-chloroamphetamine, 3,4-methylenedioxymethamphetamine, methylphenidate (ritalin), and 5-methoxy-6-methyl-2-aminoindan. For each cell line expressing a single biogenic amine

transporter, efflux of the accumulated substrate was stimulated by amphetamine derivatives, and this efflux was blocked by mazindol, an inhibitor of all three transporters. Of the amphetamine derivatives tested, some caused efflux at concentrations similar to those that inhibited transport. Other derivatives were much less effective at stimulating efflux than at inhibiting uptake. Methylphenidate caused little or no efflux, although it blocked uptake mediated by both NET and DAT. Other inhibitors of transport, such as cocaine, mazindol, citalopram, and nisoxetine, failed to stimulate efflux from these cells at concentrations that inhibited influx. The results suggest that potency toward individual plasma membrane biogenic amine transporters and the ability to release accumulated amine substrates are independent properties of each amphetamine derivative.

The biogenic amine transporters are responsible for reuptake of 5-HT, NE, and DA released by nerve endings, a process that inactivates the synaptic action of these neurotransmitters (1). These transporters are the biological targets for stimulants such as cocaine (2) and amphetamines (3, 4), as well as the sites of antidepressant action (5). The mechanism of neuronal amine release induced by amphetamine derivatives has been known for many years to be independent of extracellular Ca²⁺ and, therefore, unlikely to involve exocytosis (6). Two molecular sites of action for amphetamines have been identified, and they are the plasma membrane transporters and the vesicular storage apparatus. Recent studies with platelet plasma membrane vesicles have provided evidence that amphetamine and its derivatives interact with the SERT as substrates and that these compounds induce 5-HT release in an exchange reaction catalyzed by the transporter (7-10).

The study of biogenic amine transporters has been hindered by the lack of suitable model systems. Although platelets are a source of membranes containing the SERT (11, 12), no comparable peripheral tissue exists for NET and DAT. With the recent molecular cloning of cDNAs encoding SERT, NET, and DAT (13-20), it has become possible to examine each of the three transporters separately in heterologous expression systems. We have generated cell lines stably expressing SERT, NET, and DAT (LLC-SERT, LLC-NET, and LLC-DAT, respectively) from LLC-PK₁ cells transfected with the corresponding cDNAs (21). These cell lines demonstrate plasma membrane transport properties characteristic of each transporter, in the same cell type. Thus, the three transporters can be compared directly without complications arising from variation in source tissue.

In the work described here, we used these cell lines to investigate the specificity and mechanism of amphetamine and its derivatives acting on plasma membrane biogenic amine transporters. These compounds are thought to interact with the DAT and the NET, as well as the SERT, by

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; NE, norepinephrine; DA, dopamine; SERT, 5-hydroxytryptamine transporter; NET, norepinephrine transporter; DAT, dopamine transporter; MDMA, (+)-3,4-methylenedioxymethamphetamine; METH, (+)-methamphetamine; PCA, *p*-chloroamphetamine; MMAI, 5-methoxy-6-methyl-2-aminoindan; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; MPP⁺, 1-methyl-4-phenylpyridinium.

stimulating efflux of the endogenous substrate through a process of exchange (3, 4, 22–25). In addition, they may interact with biogenic amine storage mechanisms in secretory granules (7–10, 26, 27). In this study, we found differences between the plasma membrane transporters in their sensitivity to inhibition by various amphetamines. We also demonstrated amphetamine-induced substrate efflux from cells expressing each transporter. These results further define the specificity of each transporter and demonstrate the difference between inhibitors like mazindol and cocaine and alternative substrates like amphetamine and its derivatives.

Experimental Procedures

Methods

Cell culture. The LLC-SERT, LLC-NET, and LLC-DAT cell lines (21) were maintained in α -modified Eagle's medium, supplemented with 10% fetal bovine serum and 2 mM L-glutamine, at 37° in 5% CO₂. G418 (GIBCO) was added at a concentration of 1.8 g/liter.

Transport assay. Cells were grown in 24-well plates at 37° until they were confluent (or for at least 48 hr after plating). After one wash with 1 ml of assay buffer [PBS (28) supplemented with 1 mM MgCl₂ and 0.1 mM CaCl₂], cells were incubated in 0.2 ml of assay buffer containing ³H-labeled substrate and other inhibitors (as described in the figure legends), for a predetermined period of time at 22°. At the end of the incubation, cells were washed three times with 1 ml of ice-cold PBS and then dissolved in 0.25 ml of 1% SDS solution. The amount of substrate accumulated in the cells was determined by counting in 3 ml of Optifluor (Packard Instrument Co., Downers Grove, IL), in a Beckman LS-3801 liquid scintillation counter. L-Ascorbic acid (50 μ M) was added to DA solutions for stability. This concentration of ascorbate did not inhibit substrate uptake by either LLC-DAT or LLC-NET cells.

Efflux. Cells grown in 24-well plates as described above were incubated with radiolabeled substrate (³H]5-HT for LLC-SERT cells and ³H]MPP⁺ for LLC-NET and LLC-DAT cells) for 30 min. Substrate accumulation was then terminated, and efflux was initiated by washing of each well with 1 ml of substrate-free assay buffer and addition of 0.2 ml of assay buffer containing the indicated concentration of an amphetamine derivative or inhibitor, or both. After 30 min at 22°, cells were washed three times with 1 ml of ice-cold PBS and then dissolved in 0.25 ml of 1% SDS solution. The amount of substrate remaining in the cells was determined by counting in Optifluor as described above.

Data analysis. Nonlinear regression fits of experimental and calculated data were performed with Origin (MicroCal Software, Northampton, MA), which uses the Marquardt-Levenberg, nonlinear, least-squares, curve-fitting algorithm.

Substrate stability. To determine the stability of accumulated substrates, cells were incubated under normal transport assay conditions in the presence of ³H-labeled DA, 5-HT, or MPP⁺ for 0–60 min. The medium was removed, and the cells were washed three times with 1 ml of ice-cold PBS and then dissolved in 0.25 ml of 1% SDS solution. Samples of the medium and the cell extract were analyzed by HPLC, using a 0.46- × 25-cm C₁₈ column (Ultrasphere) for ³H]5-HT and ³H]DA and a 0.46- × 25-cm C₈ column (Partisil 10) for ³H]MPP⁺. The eluting buffer for ³H]5-HT was 20% methanol/80% 50 mM sodium acetate containing 0.01% triethylamine and 1 mM EDTA. For ³H]DA the column was eluted with 10% methanol/90% 0.2 M sodium phosphate, pH 3. ³H]MPP⁺ was eluted from the C₈ column with a gradient starting at 20% acetonitrile/80% 0.1 M sodium acetate, pH 4.5, containing 0.1% triethylamine, and changing to 95% 0.1 M sodium acetate, pH 4.5, containing 0.1% triethylamine/5% acetonitrile containing 0.1% triethylamine, over a period of 5 min. Each column was eluted at a rate of 1 ml/min. Fractions of 0.5 ml were collected and counted in Optifluor.

Materials

LLC-SERT, LLC-NET, and LLC-DAT cell lines, expressing the cloned SERT, NET, and DAT, respectively, were derived by calcium phosphate transfection of LLC-PK₁ cells, as described previously (21). The radiolabeled substrates [³H]DA, [³H]MPP⁺, and [³H]5-HT were purchased from DuPont-NEN Research Products (Boston, MA). MDMA was supplied by the National Institute on Drug Abuse. Cocaine, (+)-amphetamine, METH, and methylphenidate (racemic) were purchased from Sigma Chemical Co. (St. Louis, MO). Mazindol was purchased from Research Biochemicals (Natick, MA). MMAI (racemic) (29) was generously donated by Dr. David Nichols (Purdue University, West Lafayette, IN), nisoxetine was a kind gift from Dr. David Wong (Lilly Research Labs, Indianapolis, IN), and citalopram was graciously provided by Dr. John Hyttel (H. Lundbeck, Copenhagen, Denmark). All other reagents were purchased from commercial sources.

Results

Accumulation of MPP⁺ by cells expressing biogenic amine transporters. To examine efflux of biogenic amines that have accumulated within transfected cells, it is necessary to demonstrate that the amines are stable intracellularly. Preliminary experiments indicated that, whereas 5-HT was stable within LLC-SERT cells, DA taken up by LLC-DAT and LLC-NET cells was not stable. After taking up [³H]DA for approximately 30 min, these cells spontaneously began to lose accumulated radiolabel (data not shown). As an alternative substrate for the catecholamine transporters, we tested MPP⁺, the oxidized metabolite of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (30). Fig. 1 shows data for accumulation of [³H]MPP⁺ by each cell type. LLC-NET cells took up MPP⁺ most avidly, whereas transport into LLC-SERT cells required higher MPP⁺ concentrations. As reported and discussed previously (21), the DAT in LLC-DAT cells is more active than the NET in LLC-NET cells, in both V_{max} and turnover number. More importantly, MPP⁺ was not degraded by transfected LLC-PK₁ cells, at least in the time necessary for efflux experiments, as discussed below. LLC-NET and LLC-DAT cells accumulated [³H]MPP⁺ to a maximum that remained constant with time (data not shown). Fig. 2 shows a summary of HPLC analyses of [³H]DA, [³H]5-HT, and [³H]MPP⁺ incubated with LLC-DAT, LLC-SERT, and LLC-NET cells, respectively. With time, [³H]DA incubated with LLC-DAT cells was degraded, and a new peak of radioactivity appeared in the chromatogram. This degradation was not blocked with 90 μ M pargyline, an inhibitor of monoamine oxidase (data not shown). The fraction of radiolabel eluted with the DA peak decreased with incubation time (Fig. 2), whereas the fractions of label associated with the 5-HT peak (LLC-SERT cells) and the MPP⁺ peak (LLC-NET cells) were relatively constant. Likewise, in transport experiments internal [³H]MPP⁺ was maintained over long incubations with LLC-NET and LLC-DAT cells under conditions where internal radiolabel derived from [³H]DA would have declined dramatically (data not shown). The stability of intracellular MPP⁺ in these cells may be due, in part, to its uptake by mitochondria (30), although essentially all of the accumulated MPP⁺ was available for release, as shown below (see Fig. 4, B and C).

Inhibition of transport by amphetamine derivatives.

Fig. 3 shows data from representative experiments testing the potency of transport inhibition by a variety of amphet-

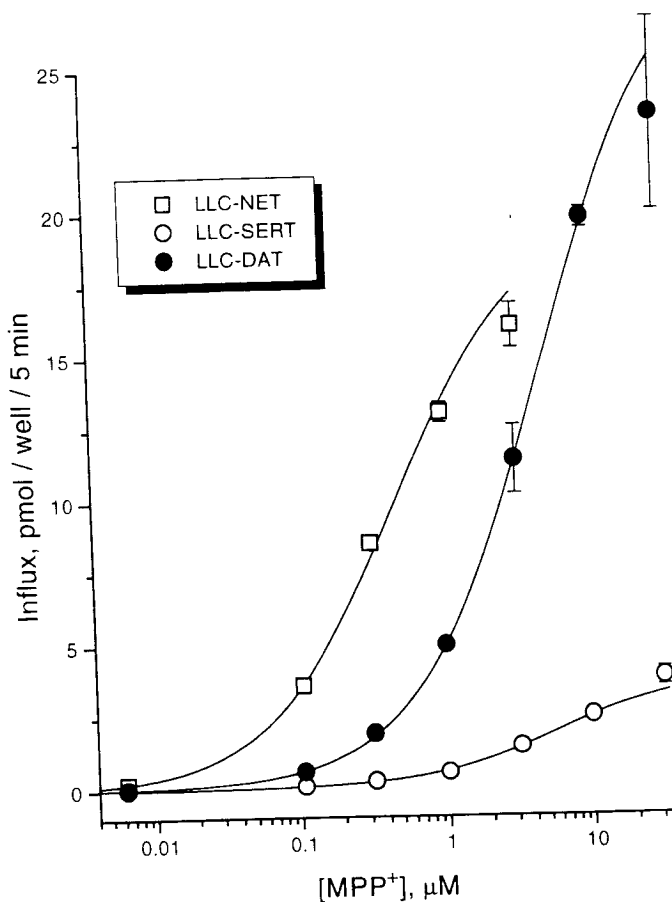


Fig. 1. Accumulation of MPP^+ by LLC-SERT, LLC-NET, and LLC-DAT cells. MPP^+ uptake rates were measured in 2-min incubations at room temperature with the indicated concentrations of $[^3H]MPP^+$, in LLC-NET, LLC-DAT, and LLC-SERT cells.

amine derivatives. $[^3H]MPP^+$ was used as a substrate for LLC-NET and LLC-DAT cells. $[^3H]5-HT$ was used with LLC-SERT cells. Using 2-min incubations as an indication of initial rate (21), we determined the potency of each inhibitor for inhibition of transport. The results demonstrate the difference in inhibitor selectivity between the catecholamine transporters (NET and DAT) and SERT. Among the most potent inhibitors of 5-HT transport into LLC-SERT cells (Fig. 3A) was MMAI, a nontoxic amphetamine derivative (29) that we have shown previously to interact strongly with SERT in platelet membranes but not with DAT in rat striatal membranes or transfected CV-1 cells (9). In the experiments shown in Fig. 3, the three transporters were compared in the identical environment of LLC-PK₁ cells. Whereas MMAI inhibited NET (Fig. 3B) and DAT (Fig. 3C) in this system, it was the least potent of the compounds tested. Amphetamine and METH were the most potent inhibitors of NET- and DAT-mediated transport but were considerably weaker inhibitors of SERT-mediated transport than was MMAI. MDMA and PCA, both of which cause destruction of serotonergic nerve endings *in vivo*, were potent inhibitors of all three transporters. The only characteristic that distinguished NET from DAT in these experiments was the higher potency of methylphenidate for inhibiting transport into LLC-DAT cells. Otherwise, the two catecholamine transporters had remarkably similar inhibition patterns, consistent with their similar substrate selectivities (21).

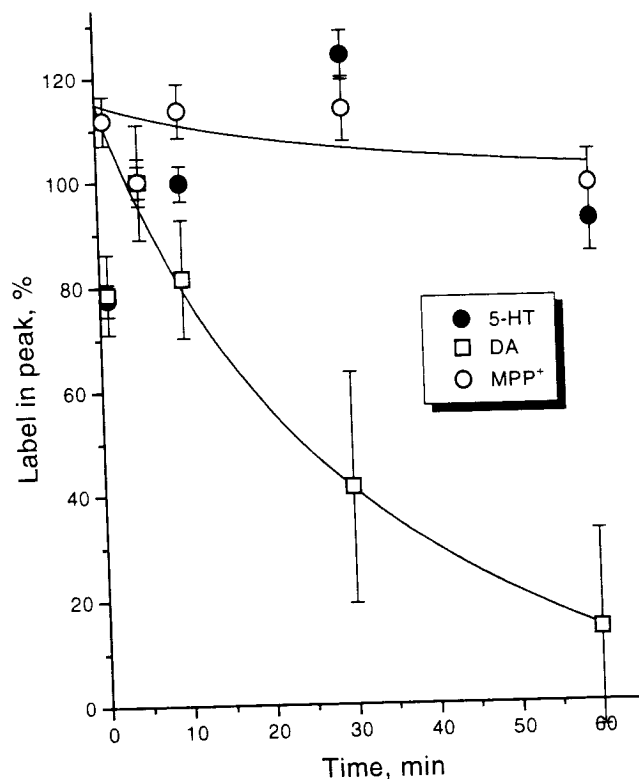


Fig. 2. Degradation of DA, 5-HT, and MPP^+ by transfected LLC-PK₁ cells. Stability of substrates in transfected cells was determined as follows. Cells were incubated with radiolabeled transport substrates under normal transport assay conditions. $[^3H]DA$ was incubated with LLC-DAT cells ($\square$), $[^3H]MPP^+$ with LLC-NET cells ($\circ$), and $[^3H]5-HT$ with LLC-SERT cells ($\bullet$). After the time indicated, a sample of the supernatant medium was taken for analysis by HPLC, as described in Experimental Procedures. The fraction of radiolabel coeluting with authentic substrate is plotted as a percentage of the total, with each time course curve normalized to 100% at 5 min to allow better comparison between substrates.

Stimulation of transporter-mediated substrate efflux by amphetamine derivatives. The experiments shown in Fig. 3 are uptake rate measurements and do not assess release of previously accumulated substrate. Because amphetamine and its derivatives are known to cause transmitter release from monoaminergic neurons (3, 4), we tested their ability to release accumulated $[^3H]MPP^+$ and $[^3H]5-HT$ from LLC-PK₁ cells stably transfected with the transporters. To measure release, we incubated cells with radiolabeled substrate for 30 min and then removed the medium and replaced it with buffer containing an amphetamine derivative or mazindol, an inhibitor of transport into each of these cell lines (21), or both. Fig. 4 shows the time course of $[^3H]5-HT$ and $[^3H]MPP^+$ efflux from LLC-SERT, LLC-NET, and LLC-DAT cells under these conditions. When the assay buffer contained an amphetamine derivative, radiolabel was lost from the cells more rapidly than under control conditions (buffer alone). Accordingly, MDMA increased efflux of $[^3H]5-HT$ from LLC-SERT cells (Fig. 4A), and amphetamine and METH increased efflux of $[^3H]MPP^+$ from LLC-NET cells (Fig. 4B) and LLC-DAT cells (Fig. 4C), respectively.

In addition, we frequently observed faster efflux when mazindol was added to the efflux medium, compared with the control, although this was always slower than efflux in the presence of an appropriate amphetamine derivative. Mazin-

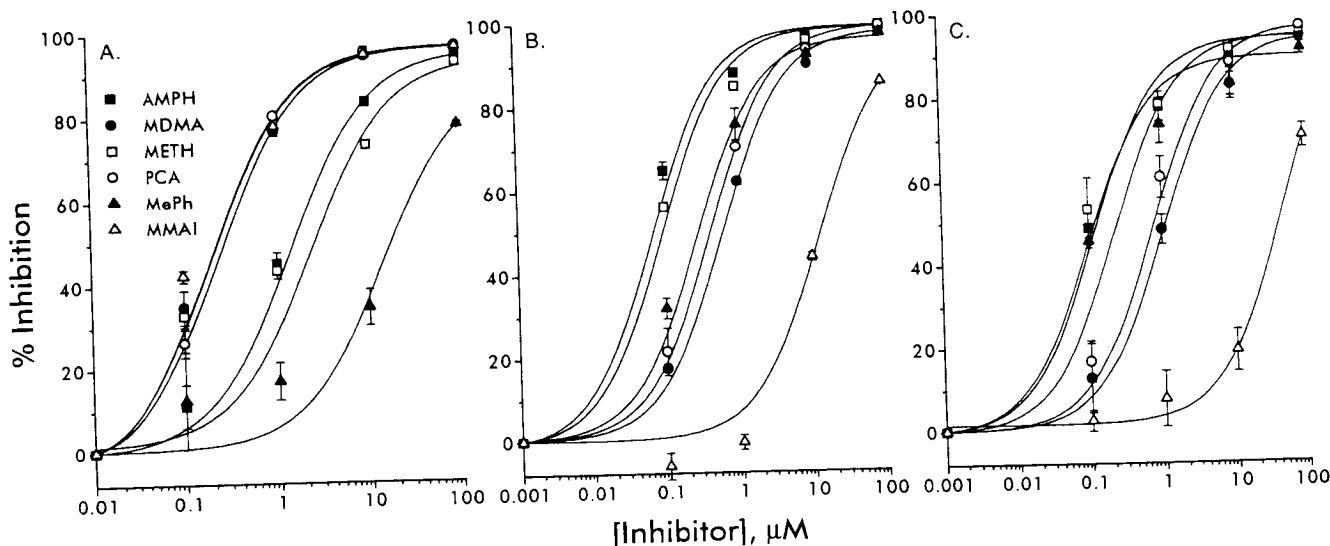


Fig. 3. Inhibition of biogenic amine uptake by amphetamine derivatives in LLC-SERT (A), LLC-NET (B), and LLC-DAT (C) cells. Uptake rates for $[^3\text{H}]\text{5-HT}$ (A) and $[^3\text{H}]\text{MPP}^+$ (B and C) were measured in 4-min incubations at room temperature, with the indicated concentrations of the following compounds: amphetamine (AMPH), MDMA, METH, PCA, methylphenidate (MePh), and MMAI.

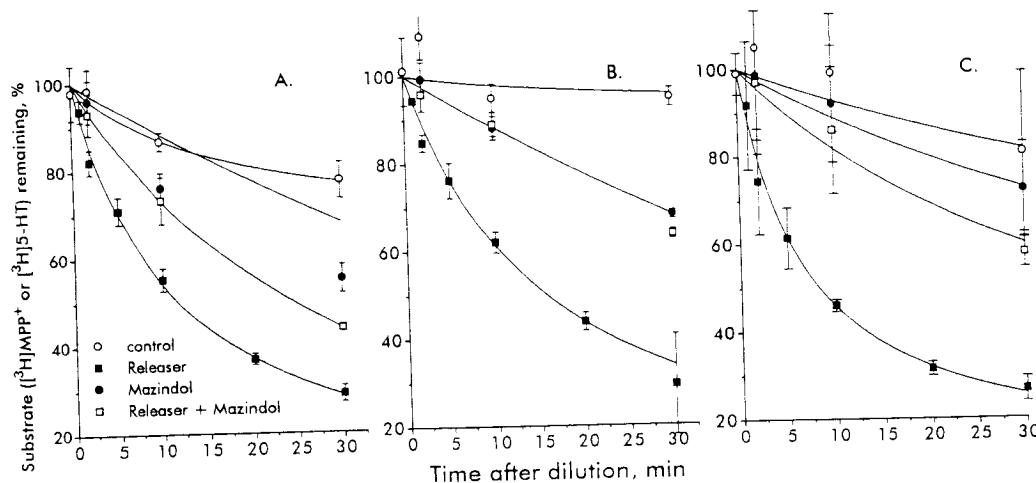


Fig. 4. Time course of biogenic amine efflux from LLC-SERT (A), LLC-NET (B), and LLC-DAT (C) cells. Cells were allowed to accumulate $[^3\text{H}]\text{5-HT}$ (A) or $[^3\text{H}]\text{MPP}^+$ (B and C) for 30 min. The external substrate was removed by dilution with fresh medium containing the following releasers: 100 μM MDMA (A), 50 μM amphetamine (B), and 100 μM METH (C) and 50 μM mazindol, as indicated. After the indicated time at room temperature, cells were washed and counted to determine remaining internal radiolabel.

dol-induced efflux is likely to result from leaks that are normally offset by reaccumulation. In the presence of mazindol, however, reaccumulation is blocked and the increased efflux under these conditions may reflect the rate of nonmediated efflux from the cells. More significantly, amphetamine-stimulated efflux was always decreased by the presence of mazindol. Thus, the amphetamine-induced increase in efflux was mediated by the transport system in each case. We found that cocaine, mazindol, nisoxetine, and citalopram failed to cause significant efflux from LLC-SERT, LLC-NET, and LLC-DAT cells even at concentrations that inhibited transport by approximately 80%. Table 1 shows the amount of efflux induced by amphetamine derivatives and inhibitors added at concentrations that inhibited approximately 80% of the initial transport rate.

It is evident from Table 1 that, for each transporter, some compounds stimulated efflux, whereas others did not. Table 1 reports measurements of efflux at drug concentrations that inhibited transport by approximately 80%. Thus, differences in potency between test compounds did not influence the measurement of efflux stimulation. For LLC-NET cells, cocaine, mazindol, methylphenidate, and nisoxetine caused efflux of approximately 26% of the internal $[^3\text{H}]\text{MPP}^+$ in the

measured time period. Amphetamine and its derivatives MDMA, METH, PCA, and MMAI all increased efflux to 33–62%. MMAI was effective at releasing internal MPP^+ even though its potency was low, relative to that of amphetamine. In the case of LLC-DAT cells, amphetamine, MDMA, METH, and PCA all caused release of MPP^+ , whereas MMAI and methylphenidate were similar to the inhibitors cocaine and mazindol. In LLC-SERT cells, PCA, MDMA, and MMAI all caused more efflux than did inhibitors (citalopram and mazindol) or amphetamine and some of its derivatives (METH and methylphenidate), whereas cocaine caused slightly more efflux than did amphetamine, although significantly less than did PCA, MDMA, or MMAI. This stimulatory effect of cocaine was not observed in other experiments, and we consider cocaine to be among the compounds that do not release internal 5-HT.

The concentration dependence for efflux induced by amphetamine derivatives shows some significant and interesting differences, compared with the inhibition of transport by the same compounds. Fig. 5 shows the extent of efflux induced in each of the cell lines in response to the same amphetamine concentration range as shown in Fig. 3 for uptake inhibition. We consistently observed maximal efflux at inter-

TABLE 1

Efflux-inducing ability of inhibitors and amphetamines, relative to transport inhibition

At a concentration that inhibited transport by 80%, each compound was tested for stimulation of [3 H]MPP $^+$ efflux from LLC-NET and LLC-DAT cells and [3 H]5-HT efflux from LLC-SERT cells. The percentage of cellular radiolabel lost within 30 min after substrate removal is given for each compound with each cell line. Zero efflux was set as the amount released in a parallel incubation with no test compound added.

	Radiolabel lost		
	NET	DAT	SERT
		%	
Amphetamine	58 $\pm$ 4	48 $\pm$ 5	7 $\pm$ 3
Citalopram	— ^a	—	-0.3 $\pm$ 2
Cocaine	25 $\pm$ 3	7 $\pm$ 3	12 $\pm$ 2
Mazindol	26 $\pm$ 2	4 $\pm$ 11	6 $\pm$ 2
MDMA	57 $\pm$ 5	39 $\pm$ 3	23 $\pm$ 3
METH	62 $\pm$ 4	46 $\pm$ 4	11 $\pm$ 6
Methylphenidate	25 $\pm$ 3	8 $\pm$ 2	5.5 $\pm$ 2
MMAI	48 $\pm$ 3	-12 $\pm$ 2	18
Nisoxetine	27 $\pm$ 2	—	—
PCA	33 $\pm$ 2	37 $\pm$ 4	28

^a —, Untested combinations of cell lines and compounds.

mediate concentrations of some compounds and less efflux at the highest concentrations tested. For LLC-SERT cells, this is apparent for PCA and MMAI (Fig. 5A). For LLC-NET and LLC-DAT cells (Fig. 5, B and C), the effect was most pronounced with METH, although other compounds showed similar (although less dramatic) inhibition at high concentrations. Methylphenidate was among the best inhibitors of MPP $^+$ uptake by LLC-DAT cells but was almost ineffective at stimulating efflux from the same cells. In LLC-NET cells, methylphenidate was approximately equipotent with MDMA for transport inhibition but was much weaker at stimulating efflux. For the compounds that stimulated efflux, the rank order of potency for each cell type was similar for inhibition of transport (Fig. 3) and stimulation of efflux (Fig. 5).

Discussion

The results presented here define the specificity of amphetamine and its derivatives for their actions on biogenic amine transporters. These compounds inhibit transport into, and stimulate substrate efflux from, cells stably transfected with DAT, NET, and SERT. Use of cells stably expressing the cloned transporters allowed us to study each of the transporters independently in the same cell type, allowing a degree of comparison previously unavailable. We observed differences in amphetamine specificity between the transporters, both in the relative potencies of the compounds and in their abilities to stimulate substrate efflux.

Although this experimental system allows comparison between the three transporters, we must note that differences between the LLC-PK $_1$ cell line and aminergic neurons might modify the properties of transporters expressed in this heterologous system. It is possible that differences in post-translational processing or transporter-associated proteins specific to neurons may alter the amphetamine specificity of a transporter. For example, Pifl *et al.* (31) noted that the substrate specificity of DAT varied depending on the cells in which it was expressed. We assume, although we cannot be certain, that the properties measured here reflect the *in vivo* specificities for the three transporters.

As expected, we observed differences between the transporters in their sensitivity to inhibition by amphetamine derivatives. [3 H]5-HT transport into LLC-SERT cells was most sensitive to PCA, MDMA, and MMAI. Amphetamine and METH were approximately 20 times less potent, and methylphenidate was the weakest of the compounds tested. Cells expressing DAT had the opposite specificity. [3 H]MPP $^+$ transport into LLC-DAT was most sensitive to methylphenidate, amphetamine, and METH. PCA and MDMA were about 10-fold less potent, and MMAI was >20-fold less potent than MDMA. [3 H]MPP $^+$ transport into LLC-NET cells demonstrated a pattern of amphetamine inhibition very similar to that seen with LLC-DAT cells. The only reproducible difference in the inhibition of NET and DAT was their sensitivity to methylphenidate, which was less potent in the LLC-NET cells than in the LLC-DAT cells. This similarity between LLC-NET and LLC-DAT cells reflects their similar substrate specificities, in contrast to their distinct inhibitor sensitivities (21). We take this as support for the proposal that amphetamine derivatives interact with biogenic amine transporters as substrates rather than as inhibitors.

Because the cell lines used in this work do not contain vesicles competent for amine storage and secretion, amphetamine effects on these cells do not reflect any interaction with secretory vesicles or their transport systems. However, the specificity of amphetamine derivatives arises, in large part, from their interactions with plasma membrane transporters. Thus, although the cell lines used in the present work do not shed light on the interactions of amphetamine derivatives with synaptic vesicles, they provide important information about the interactions of those compounds with the plasma membrane transporters that determine their cellular specificity.

We observed previously that amphetamine derivatives varied in their ability to interact specifically with the vesicular monoamine transporter of chromaffin granules (10). We found that all of the amphetamines tested dissipated the transmembrane pH difference (Δ pH) but that some compounds (MDA, MDMA, and fenfluramine) competed with [3 H]reserpine binding, whereas others (PCA) did not. Peter *et al.* (32) observed that the two rat isoforms of the vesicular monoamine transporter had different sensitivities to METH in a heterologous expression system. Taken together with the differences observed here between plasma membrane transporters, the data suggest that amphetamine-transporter interactions are sufficiently complex to account for the wide range of physiological effects observed with different amphetamine derivatives.

With a few notable exceptions, each amphetamine derivative caused efflux from cells expressing SERT, NET, and DAT. We interpret this as support for the idea that inhibition of influx and stimulation of efflux represent the same basic phenomenon. Amphetamine derivatives act as substrates for plasma membrane biogenic amine transporters (7–10, 33) and, as substrates, these compounds compete with other substrates for influx and also stimulate efflux of internal substrates by transporter-mediated exchange. An unexpected finding was that the efflux caused by amphetamines reached a maximum and then decreased with additional increases in concentration. The effect was apparent for each of the three cell lines, although not with the same compounds for each system. High concentrations of 5-HT are thought to

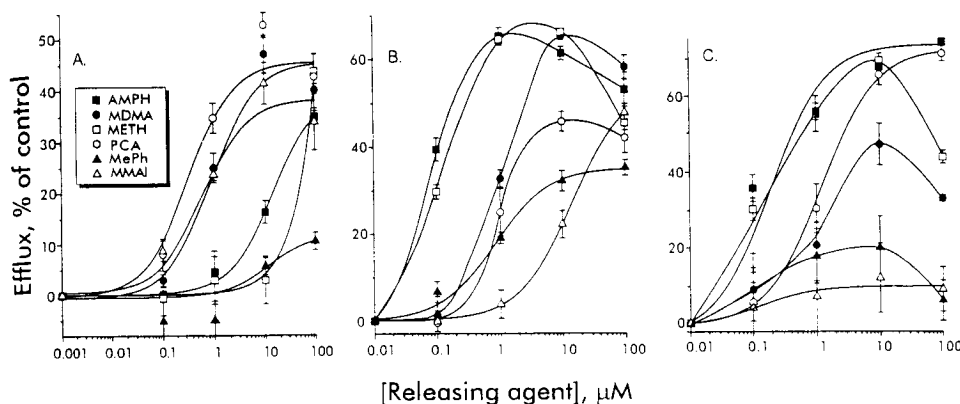


Fig. 5. Concentration dependence for biogenic amine efflux from LLC-SERT (A), LLC-NET (B), and LLC-DAT (C) cells. Cells were allowed to accumulate [3 H]-HT (A) or [3 H]MPP $^+$ (B and C) for 30 min. The external substrate was removed by dilution with fresh medium containing the indicated concentration of the following releasing agents: amphetamine (AMPH), MDMA, METH, PCA, methylphenidate (MePh), and MMAI. After 30 min at room temperature, the cells were washed and counted to determine remaining internal radiolabel. Efflux was calculated as the percentage of radiolabel lost from the cells, relative to the control (dilution into medium containing no releasing agent).

bind at an additional low affinity site that influences imipramine dissociation (34–36). It is possible that the inhibition of efflux observed at high amphetamine concentrations is due to an inhibitory action at this low affinity site or at analogous sites in the NET and DAT.

It is interesting to compare our results with those of Eshleman *et al.* (25), using DAT expressed transiently in COS-7 cells. In those cells, unlike LLC-PK $_1$ cells, degradation of [3 H]DA was reportedly not a problem. The ability of METH to induce [3 H]DA release mirrored our results on [3 H]MPP $^+$ release from LLC-DAT cells, in both its effectiveness and its potency. However, Eshleman *et al.* (25) observed that certain drugs, such as GBR-12935 and mazindol, decreased background [3 H]DA release, whereas others, including cocaine, had no effect. We observed no consistent differences between cocaine and mazindol with any of the stably transfected cell lines (Table 1).

Amphetamine and its derivatives apparently vary not only in their relative potency towards biogenic amine transporters but also in their ability to stimulate efflux. Binding of these compounds to the active site of a transporter inhibits its catalytic activity but does not necessarily promote efflux of internal substrate. Additionally, efflux stimulation seems to require that the amphetamine derivative is transported into the cell, converting the transporter into a form that binds internal substrate (1). Thus, inhibitory potency and the ability to stimulate efflux appear to be independent properties of this class of drugs.

Of particular interest is the relative lack of efflux induced by methylphenidate. Although this compound is the most potent inhibitor of [3 H]MPP $^+$ influx into LLC-DAT cells, it is one of the weakest compounds at stimulating [3 H]MPP $^+$ efflux. Likewise, efflux from LLC-NET cells was much weaker with methylphenidate than with MDMA, PCA, amphetamine, or METH. With LLC-SERT cells also, methylphenidate caused little efflux, even at concentrations that caused significant inhibition of uptake. These results are consistent with a previous report (37) that methylphenidate failed to deplete brain monoamines when administered *in vivo*. The fact that methylphenidate inhibits transport without stimulating efflux suggests that, even within the group of compounds structurally resembling amphetamine, there are structural requirements for transport that do not affect binding.

Acknowledgments

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