Stable Expression of Biogenic Amine Transporters Reveals Differences in Inhibitor Sensitivity, Kinetics, and Ion Dependence*

(Received for publication, August 11, 1993, and in revised form, December 6, 1993)

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We have constructed stable cell lines expressing transporters for dopamine (DA), norepinephrine (NE), and serotonin (5-HT) by transfection with cloned cDNAs. The parental LLC-PK1 cell does not express any of these neurotransmitter transporters. Therefore, monoamine transport activities in each of these cell lines are due to the transfected DNA only, allowing comparison in the same background. Drug inhibition profiles for each cell line are distinct and as expected for each transporter. LLC-NET and LLC-DAT cells transported both NE and DA and both cell types exhibited a lower K_i for DA transport than for NE transport. Analysis of V_max data for LLC-NET cells suggests that substrate is bound to the NE transporter during the rate-limiting step(s) in transport. The cocaine analog 2-β-carbomethoxy-3β-(4-[125]I)iodophenyl)tropane binds to each cell type, and is displaced by transport substrate in each case. Binding and transport measurements on parallel cell cultures allowed estimation of turnover numbers for norepinephrine, dopamine, and serotonin transporters. All three transporters require external Na^+ and Cl^- ions. The Na^+ concentration dependence suggests that a single Na^+ ion is involved in transport catalyzed by norepinephrine and serotonin transporters while more than one Na^+ ion participate in transport mediated by the dopamine transporter.

The synaptic action of neurotransmitters released by nerve cells is terminated by a re-uptake process in which the transporters are pumped back inside the nerve endings from which they were released. Recently, cDNAs encoding transporters for neurotransmitters, amino acids, and other substrates have been cloned. These include transporters for the biogenic amines norepinephrine (NA),1 dopamine (DA), and serotonin (5-HT) (1–8). Many of these transporters share extensive sequence homology and constitute a multigene family (10, 11). In addition to this structural similarity, these transporters share a functional dependence on Na^+ and Cl^- ions (12–17). In some cases, this dependence has been shown to reflect the cotransport of Na^+ and Cl^- with the neurotransmitter substrate (18–21). Drugs that modulate the activity of the biogenic amine transporters produce profound behavioral effects, leading to their therapeutic use in depression, obsessive-compulsive disorder, and other mental diseases (22–25) and also to their abuse as psychotropic agents (26–28).

Of the biogenic amine transporters, the 5-HT transporter is arguably the best understood, largely due to studies with plasma membrane vesicles from human platelets (18, 19, 21, 29, 30) and placenta (31–33). Evidence suggests that extracellular 5-HT binds to the transporter together with one Na^+ ion and one Cl^- ion. After translocation of these solutes, and their dissociation to the cytoplasm, the transporter binds a K^+ ion and transports it to the external face of the membrane. This reaction cycle, which is likely to be rate-limiting in the overall reaction cycle, regenerates the form of the transporter capable of binding another molecule of external 5-HT. Not as much information is available for the NE and DA transporters, although the NE transporter has been studied in membrane vesicles from placenta (15) and PC-12 cells (34, 35). Estimates of the transport stoichiometry of DA and NE transport have come mainly from measurements of initial rates of transport and its ion dependence. For NE transport into placental brush-border membrane vesicles (15) and intact PC-12 cells (36), initial rates of transport showed a simple hyperbolic dependence on Na^+ or Cl^-, consistent with a Na^+:Cl^- stoichiometry of 1:1. The DA transporter, however, has a different ion dependence. While initial rates of DA transport were found to be dependent on a single Cl^- ion, two Na^+ ions were apparently involved in the transport process (37). Thus, the initial rate of DA transport into suspensions of rat striatum was a simple hyperbolic function of [Cl^-] but depended on [Na^+] in a sigmoidal fashion. These data are consistent with a Na^+:Cl^- stoichiometry of 2:1.

In these studies and others, the transporters were studied in diverse preparations including brain slices, synaptosomes, platelet, and placental membrane vesicles and cultured cells. The differences that have been noted in transporter properties in these preparations (including substrate specificities, transport kinetics, ion dependence, and inhibitor sensitivities) may be due, in some cases, to the different cell backgrounds or tissue origins, and in other cases to real differences between transporters. Furthermore, the possibility that more than one transport system is present in a given preparation adds additional uncertainty. The cloning of transporter cDNAs allows a more specific approach to studying transporter function. Here we report the generation and characterization of stable cell lines expressing individual transporters for DA, NE, and 5-HT. The parental LLC-PK1 cell does not express any of these neurotransmitter transporters. Therefore, the transport activities in each of these cell lines are due to the inserted transporter cDNA only, and the transporters can be compared in the same background. These stable transfected cell lines exhibited differences in transport kinetics, ion dependence, inhibitor sensitivities, ligand binding affinities, and turnover numbers.

EXPERIMENTAL PROCEDURE

Materials—LLC-PK1 cells were a gift from Dr. Michael Caplan, Department of Cell and Molecular Physiology, Yale University. The cDNA encoding the rat DA transporter (DAT) was a generous gift from Dr. Michael Caplan. The cloning of transporter cDNAs allows a more specific approach to studying transporter function. Here we report the generation and characterization of stable cell lines expressing individual transporters for DA, NE, and 5-HT. The parental LLC-PK1 cell does not express any of these neurotransmitter transporters. Therefore, the transport activities in each of these cell lines are due to the inserted transporter cDNA only, and the transporters can be compared in the same background. These stable transfected cell lines exhibited differences in transport kinetics, ion dependence, inhibitor sensitivities, ligand binding affinities, and turnover numbers.

* This work was supported by United States Public Health Services Grants DA 8213 and DA 7299. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: NA, norepinephrine; DA, dopamine; 5-HT, serotonin; β-CIT, 2-β-carbomethoxy-3β-(4-[125]I)iodophenyl)tropane; DAT, DA transporter; CMV, cytomegalovirus; α-MEM, α-modified Eagle's medium; FBS, fetal calf serum; PBS, phosphate-buffered saline; SERT, serotonin transporter; NET, norepinephrine transporter.
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George Uhl, NIDA Addiction Research Center, Baltimore, MD. NET cDNA, encoding the human NE transporter, was graciously supplied by Dr. Susan Amara, Vollum Institute, Portland, OR. SERT cDNA, coding for the rat 5-HT transporter, was kindly contributed by Dr. Beth Hoffman, National Institutes of Mental Health, Bethesda, MD. Vector pUC-CMV was purchased from Invitrogen (San Diego, CA). Radiolabeled serotonin ([3H]5-HT), dopamine ([3H]dopamine), NE ([3H]NE), and [125I]p-CIT were purchased from Du Pont New England Nuclear Research Products. Ligand ([125I]p-CIT (2200 Ci/mmole), generously provided by Dr. Ron Baldwin, was synthesized as previously described for [125I]p-CIT (38). All other reagents were purchased from commercial sources.

Cell Culture—The parental LLC-PK1 cells were maintained in α-modified Eagle’s medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37 °C, 5% CO2. The transfected cell lines were maintained in the same medium except that G418 (Gibco) was added at a concentration of 1.8 μg/ml. In some cases, α-MEM + FBS was replaced with UltraCULTURE (BioWhittaker).

Transfection—DNAs encoding the transporters were subcloned into vector pRC/CMV which has a bacteriophage T7 promoter sequence and enhancer/promoter sequences from the immediate early gene of human cytomegalovirus (CMV). A polyadenylation signal and transcription termination sequences are also present in this vector for high level expression of the inserted cDNA. Calcium phosphate-mediated transfection was performed as described (39). LLC-PK1 cells were plated in 15-cm diameter tissue culture dishes at 25% confluence and grown overnight. For each dish, about 100 μg of plasmid DNA in 4.4 ml of TE buffer (10 mM Tris–Cl, pH 7.4, 1 mM EDTA) was mixed with 9.5 μl of 2 × HBS (260 mM NaCl, 7 mM KCl, 1.5 mM MgCl2, and 0.1 mM dithiothreitol) and 4.9 μl of 10% NaHPO4, 15 mM dextrose, 50 mM HEPES, pH 7.05) in a polystyrene tube, to which 0.062 ml of 2 M CaCl2 was added dropwise while the tube was gently vortexed. The tube was incubated at room temperature for 30 min to allow a cloudy precipitate to form. The cells were washed three times with PBS (11.8 mM NaCl, 2.7 mM KCl, 1.5 mM MgCl2, and 0.1 mM dithiothreitol) and the DNA–calcium phosphate mixture was added dropwise to cover all cells. Five minutes later, 15 ml of α-MEM containing 10% FBS and 0.1 mM chloroquine was added gently to the cells covered by DNA–calcium phosphate precipitate. The dish was then incubated for 9 h at 37 °C with 5% CO2. After aspirating the medium, 15 ml of α-MEM without serum containing 15% glycerol was added to the cell and the dish was allowed to sit at room temperature for 5 min. After one wash with α-MEM, the cells were fed with 40 ml of α-MEM with 10% FBS and incubated at 37 °C. Twentyfour hours later, cells were split in α-MEM with 10% FBS into four 15-cm dishes at dilutions of 1:2, 1:5, 1:10, and 1:20. Twentyfour hours after splitting, G418 was added to the culture medium to a final concentration of 1.8 μg/ml. The culture medium was replaced with fresh medium every 3 days thereafter. Ten to 15 days later, single colonies appeared and they were picked with cloning cylinders. Clonal cell lines were obtained by limiting dilution in 96-well plates.

Transport Assay—Cells were grown in 24-well plates at 37 °C until they were confluent (or at least 48 h after plating). After one wash with 1 ml of assay buffer (PBS 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 each figure legend) for a predetermined period of time at 22 °C. At the end of the incubation, the cells were washed three times with 1 ml 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 Opti-fluor (Packard Instrument Co.) in a Beckman LS-3801 liquid scintillation counter. In ion dependence experiments, NaCl was replaced with LiCl or sodium isethionate isoosmotically. Dopamine is a better substrate for both DAT and NET and it is more stable than NE. Therefore, we used dopamine as substrate for both transporters unless otherwise stated. [3H]dopamine was used as substrate for NET in a study of saturable binding. Dopamine was a better substrate for both DAT and NET and it is more stable than NE. Therefore, dopamine was used as substrate for both transporters unless otherwise stated. Fifty μM L-ascorbic acid was added to DA and NE solutions to stabilize the amine substrates. This concentration of ascorbate did not inhibit substrate uptake by either LLC-DAT or LLC-NET cells. 5-HT was used as substrate for LLC-SERT cells.

Crude Membrane Preparation—Cells were grown to confluence as a monolayer on 15-cm tissue culture dishes. The cells were washed once with assay buffer and harvested in the same buffer (10 mM d-glucose) by scraping. Cell suspensions were homogenized on ice with a tight fitting Teflon homogenizer for 20 strokes and centrifuged in a SS-34 rotor at 20,000 rpm (48,000 × g) for 15 min at 4 °C. The pellets were suspended in ice-cold assay buffer, frozen quickly in liquid nitrogen, and stored in a −80 °C freezer.

Luciferase Binding—Crude membrane preparations were thawed on ice and diluted to a final concentration of about 0.1 μg of protein/ml in assay buffer containing 0.01 mM [125I]p-CIT and unlabeled β-CIT or transport substrates as described in figure legends. 0.5-ml aliquots of the membrane suspension were incubated at 22 °C for 30 min. Binding was stopped by addition of 4 ml of ice-cold PBS to each aliquot, and the suspensions were immediately filtered through a glass fiber filter (Schleicher & Schuell #32 Glass) followed by two washes of 4 ml of ice-cold PBS. The filters were then counted in 3 ml of Optiphase scintillation fluid. β-CIT binding on whole cells was measured essentially the same as was transport, but [125I]p-CIT was added instead of [3H]-labeled substrates and the incubation time was 30 min. β-CIT binding on whole cells showed a significant background nonspecific signal. Mazindol, a potent inhibitor for all three transporters, reduced β-CIT binding to the transfected cells to the level of the parental LLC-PK1 cells. Therefore, mazindol-sensitive β-CIT binding data was used as a measure of specific β-CIT binding to transporters on whole cells.

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

RESULTS

Generation of Stable Cell Lines Expressing the Transporters—DNAs encoding transporters for dopamine, norepinephrine, and serotonin were subcloned downstream from both T7 and CMV promoters in the eukaryotic expression vector pRC/CMV. The plasmid constructs containing each cDNA were first tested for functional activity in the vaccinia-T7 transient expression system (40) and then transfected into LLC-PK1 cells as described under "Experimental Procedures." Colonies growing in G418 containing medium were isolated and tested for transport activity. The most active ones were selected and clonal cell lines were obtained by limiting dilution. We first had some difficulty with the transfecion of serotonin transporter. We suspected that serotonin, which is present in the FBS used in culture media, might be toxic to cells that accumulate the amine by the expressed transporter. Accordingly, we replaced the normal medium (α-MEM + 10% FBS) with a serum-free medium. We were able to generate stable cell lines expressing SERT, but the cells grew poorly in the absence of serum. Surprisingly, SERT expressing cells (LLC-SERT) were not sensitive to serotonin or FBS, and they grew much better in α-MEM with 10% FBS. Fig. 1 shows the time courses of substrate accumulation by the clonal cell lines expressing DAT, NET, or SERT (LLC-DAT, LLC-NET, and LLC-SERT) and the parental LLC-PK1 cells. The stable cell lines exhibited transport activi-

Fig. 1. Time course of transport by stable cell lines. Transport assays were performed as described under "Experimental Procedures" for the indicated times using [3H]dopamine (52 nm) for LLC-NET (squares) and LLC-DAT (circles), [3H]serotonin (63 nm) for LLC-SERT (triangles), and each labeled substrate (diamonds and inverted triangles for DA and 5-HT, respectively) with LLC-PK1, the parental cell line. Each point represents the mean of three measurements and error bars represent S.E. of the mean.

25 30
05 10 Substrate uptake, pmol/mg protein

0 5 10 15 20 Time, minutes

LLC-DAT

LLC-SERT

LLC-NET

LLC-PK1

0

5

10

15

20

25

30

0

5

10

15

20

25

30

0

5

10

15

20

25

30

0

5

10

15

20

25

30

0

5

10

15

20

25

30

0

5

10

15

20

25

30

0

5

10

15

20

25

30
ties of more than 100-fold over the background of the parental cell (Fig. 1). Kinetic experiments revealed that 2-min time points were within the initial linear range, therefore, transport measurements of 2 min were used to calculate initial transport rate.

Drug Inhibition Profiles—To determine whether transport into the stable cell lines retained an inhibitor sensitivity similar to that of the native transporters, we tested five drugs for their ability to block the initial rate of biogenic amine accumulation (Fig. 2). Desipramine was the most potent transport inhibitor for LLC-NET cells, and paroxetine was the most potent for LLC-SERT cells. Mazindol inhibited each of these three cell lines at similar concentrations. Cocaine and amphetamine, while inhibiting all three transporters, were not equally effective in each case. Cocaine was most potent at inhibiting LLC-SERT cells, and least potent for LLC-NET. Amphetamine, in contrast, was most potent for LLC-NET cells and the least potent inhibitor for LLC-SERT. In these experiments, drugs and substrates were added to cells at the same time. The inhibition profiles may, therefore, not be precise reflections of the individual drug affinities, since equilibrium binding of some of these drugs may take longer than the 2-min transport measurement. In other experiments in which drugs were preincubated with cells for 2–5 min before substrates were added, there were shifts of the inhibition curves to lower concentrations (data not shown).

Transport Kinetics—Since the substrate specificity of cloned NE and DA transporters had not been investigated (1, 2, 4, 6, 7), we decided to test both \(^{3}H\)NE and \(^{3}H\)DA as substrates in LLC-NET and LLC-DAT cells. We measured the kinetics of transport for these reactions and also for \(^{3}H\)5-HT transport by LLC-SERT cells. Influx was also measured in the presence of 100 μM cocaine, which inhibits all three transporters completely (Fig. 2). The cocaine-resistant residual activity, which represents nonspecific processes (substrate binding to cells, diffusion, etc.), was subtracted to obtain net transport rates in these kinetic studies. As shown in Fig. 3, both LLC-DAT and LLC-NET cells transport both NE and DA. In both cell types, DA was the preferred substrate, with a lower \(K_M\) for transport. In LLC-DAT cells, \(V_{max}\) values for NE and DA were the same. However, The \(V_{max}\) for DA transport by LLC-NET cells was higher with DA as a substrate than with NE. The \(V_{max}\) and \(K_M\) values for LLC-DAT, LLC-NET, and LLC-SERT cells are from the same experiment.
values for all three cell types are summarized in Table I.

**Ion Dependence of Transport**—Previous studies of the serotonin transport system in human platelets and placenta show that in each transport cycle one Na⁺ ion and one Cl⁻ ion are co-transported with the substrate 5-HT, and one K⁺ ion is counter-transported (41). The DA and NE transport systems have also been shown to require external Na⁺ and Cl⁻ (12, 15–17). The effect of these ions on transport rate can provide some information about their involvement in the transport process. We therefore investigated the Na⁺ and Cl⁻ dependence of transport into all three cell lines by replacing Na⁺ and Cl⁻ with isothionate ions with equal affinities (data not shown). The cations considered for replacing Na⁺, Li⁺, and N-d-methylglucamine gave similar results, but choline inhibited transport (data not shown). We conclude that Li⁺ and isothionate were essentially inert as replacement ions for these transporters.

The rate of transport was a simple hyperbolic function of Na⁺ and Cl⁻ concentration for all cases except for the Na⁺ dependence of transport by LLC-DAT cells (Fig. 4). The sigmoidal shape of DAT-mediated transport rate versus Na⁺ concentration suggests that more than one Na⁺ are involved in this transport process. Therefore, we measured equilibrium binding of P-CIT using membranes prepared from LLC-DAT, LLC-NET, and LLC-SERT cells. From the data in Fig. 5 we calculated dissociation constant (Kᵦ) values of 3.7 nm for LLC-DAT, 8.3 nm for LLC-NET, and 0.28 nm for LLC-SERT.

**Determination of Substrate Binding Constants**—In the crude membrane preparations used for β-CIT binding, no ion gradients were imposed across the membranes, and no substrate accumulation took place. Under these conditions, the substrates still bind to the transporters, and are competitive inhibitors of equilibrium β-CIT binding. We therefore measured the ability of DA, NE, or 5-HT to inhibit β-CIT binding as a means to determine the dissociation constant (Kᵦ) for each transport substrate with its cognate transporter (Fig. 6).
binding experiments on whole cells were performed in 24-well cultures of LLC-DAT, LLC-NET, and LLC-SERT cells. P-CIT was measured transport rates and P-CIT binding to parallel plates similar to transport assays. At low concentrations, the values were calculated from the inhibition profiles shown in Fig. 6, and are listed in Table I along with the transport parameters.

**Determination of Turnover Numbers**—To determine turnover numbers for the three transporters expressed in LLC-PK₁ cells, we measured transport rates and β-CIT binding to parallel cultures of LLC-DAT, LLC-NET, and LLC-SERT cells. β-CIT binding experiments on whole cells were performed in 24-well plates similar to transport assays. At low concentrations, the amount of β-CIT binding to the transfected cells was approximately three times that of the parental LLC-PK₁ cells. Binding to the parental line represents the nonspecific processes by which β-CIT associates with cell membranes or accumulates within the cells. Nigericin inhibited a portion of the nonspecific binding, suggesting that accumulation within acidic subcellular compartments was partially responsible. Mazindol, which is a very potent inhibitor for all three transporters, had relatively little effect on LLC-PK₁ cells, but markedly inhibited β-CIT binding to cells expressing the transporters. Residual β-CIT binding, insensitive to mazindol, was about the same in transfected and parental cells. Therefore, mazindol-sensitive β-CIT binding was used to estimate the amounts of β-CIT bound specifically to the transporters.

From the $K_D$ for β-CIT (measured in membranes from each cell type) and the amount bound to intact cells at a known β-CIT concentration, we estimated the number of transporters expressed on the surface of each cell type. Likewise, from the $K_M$ for transport and the rate at a given substrate concentration, we estimated maximal transport rates. From these two values, we calculated the turnover number for each transporter (Table II). We also counted the number of cells in each well to estimate the number of accessible transporters per cell. We estimated that the average LLC-DAT cell contains 23,000 DA transporters, that 40,000 NE transporters are expressed per LLCDAT cell, and 1,000 5-HT transporters are available per LLC-SERT cell. The turnover numbers were calculated to be 115 min⁻¹ for the dopamine transporter, 6.5 min⁻¹ for the norepinephrine transporter, and 110 min⁻¹ for serotonin transporter. These data are summarized in Table II.

**DISCUSSION**

In this paper, we have described the generation of three stable cell lines expressing rat dopamine transporter, human norepinephrine transporter, and rat serotonin transporter, respectively. The common parental LLC-PK₁ cell does not transport biogenic amines. Therefore, the transport activity observed in each transfected cell line was due solely to the cDNA encoding the transporter protein. This approach overcomes some problems associated with previous studies on biogenic amine transporters in preparations such as synaptosomes (37, 44, 45) or placental membranes (15, 31) which contain more than one of these transporters. By studying each of the three transporters in the same background of LLC-PK₁ cells, differences and similarities between the transporters can be observed more reliably.

Previous studies have not agreed on the ability of the dopamine transporter to transport NE (1, 7). We found in this study that both LLC-DAT and LLC-NET cells accumulate both DA and NE. In both cell lines, the $K_M$ for DA is lower than that for NE (5.2 and 17 μM, respectively, for LLC-DAT cells, and 0.24 and 0.58 μM, respectively for LLC-NET). Furthermore, the $V_{max}$ for DA transport by LLC-NET cells is about twice that for NE.
membrane vesicles was shown to have an inhibitor sensitivity characteristic of NET (47). This transporter exhibited a much higher $K_D$ for Cl$^-$ (180 mM) than we observed here for LLC-NET cells. In contrast, 5-HT transport by LLC-SERT cells has a Na$^+$ and Cl$^-$ dependence similar to that of human platelet plasma membrane vesicles (18, 21). The turnover number for DA transport into LLC-NET cells was over 10-fold lower than that for DA transport into LLC-DAT or 5-HT transport into LLC-SERT cells. We cannot rule out the possibility that the NET cDNA contains a mutation as a result of a cloning artifact or is expressed improperly in LLC-PK1 cells. However, transient expression of NET cDNA in the vaccinia-T7 system (40) gave a Cl$^-$ sensitivity similar to that of LLC-NET cells (data not shown).

Table II

| Cells       | β-CIT binding constant $K_D$ | Specific β-CIT binding at 27 ps | Maximum β-CIT binding | Number of accessible transporters | Substrate | Maximum transport activity | Turnover numbers |
|-------------|-------------------------------|---------------------------------|-----------------------|-----------------------------------|-----------|---------------------------|------------------|
|            | pmol/mg                        |                                 |                       |                                   |           |                           |                  |
| LLC-DAT    | $3.7 \pm 0.3$                 | $0.019 \pm 0.0003$              | $2.6 \pm 0.2$         |                                   | DA        | $300 \pm 19$              | $115 \pm 16$    |
| LLC-NET    | $9.0 \pm 0.3$                 | $0.015 \pm 0.0001$              | $4.9 \pm 0.2$         |                                   | DA        | $32 \pm 1$                | $6.5 \pm 0.5$   |
| LLC-SERT   | $0.26 \pm 0.02$               | $0.010 \pm 0.0004$              | $0.11 \pm 0.01$       |                                   | 5-HT      | $12 \pm 0.4$              | $110 \pm 13$    |

Our kinetic data allow some additional conclusions about these transporters. Dopamine transport into LLC-NET cells saturates at a higher $V_{\text{max}}$ than does NE transport. This suggests that at least one step which is rate-limiting in NET-catalyzed transport involves bound substrate. In contrast, LLC-DAT cells transport both substrates with the same $V_{\text{max}}$ (300 pmol/min/mg protein). This suggests that either a different step (one in which substrate is not bound) is rate-limiting in DAT, or that the same step is rate-limiting, but the rate of that step in DAT is the same with either substrate. Table I compares the substrate dissociation constants ($K_D$), measured by β-CIT displacement, and the transport $K_M$ values. The $K_D$ values for DA and NE binding to NET, and NE binding to DAT, are 12-15 times larger than the corresponding transport $K_M$ values, while those values for DA binding to DAT and 5-HT to SERT are close to the corresponding transport $K_M$ values. To the extent that $K_D$ exceeds $K_M$, the maximal rate of transport can be reached at substrate concentrations that do not completely occupy the binding site. This may occur, for example, if the rate-limiting step in the transport cycle does not immediately follow substrate binding.

The three transfected cell lines described here provide an ideal system for comparing the ion dependence of the biogenic amine transporters. From previous studies, the transporters are known to require external Na$^+$ and Cl$^-$ for maximal activity (12-17), but each transporter has been studied in a different preparation. Furthermore, some of the preparations used contain multiple neurotransmitter transporter systems (15, 31, 37, 44, 45). We observed a simple hyperbolic dependence of substrate influx on Na$^+$ concentration for LLC-NET and LLC-SERT cells, consistent with the involvement of a single Na$^+$ ion. In contrast, the Na$^+$ dependence of DA transport into LLC-DAT cells was sigmoidal, suggesting that more than one Na$^+$ might be involved. These data were fit by assuming that two independent Na$^+$ ions are required for dopamine transport, but a more accurate estimate of cotransport stoichiometry will require additional kinetic or thermodynamic evidence.

In addition to the apparent difference in Na$^+$ stoichiometry, the LLC-NET and LLC-DAT cells also differ in their Cl$^-$ dependence. LLC-NET cells are almost 20-fold more sensitive ($K_M = 5.1 \text{ nM}$ for Cl$^-$ in LLC-NET and 100 nm in LLC-DAT). A DA and NE transport system recently characterized in placental
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