

# Quantitative Analysis of Inward and Outward Transport Rates in Cells Stably Expressing the Cloned Human Serotonin Transporter: Inconsistencies with the Hypothesis of Facilitated Exchange Diffusion

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## ABSTRACT

Quantitative aspects of inward and outward transport of substrates by the human plasmalemmal serotonin transporter (hSERT) were investigated. Uptake and superfusion experiments were performed on human embryonic kidney 293 cells permanently expressing the hSERT using [<sup>3</sup>H]serotonin (5-HT) and [<sup>3</sup>H]1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) as substrates. Saturation analyses rendered  $K_m$  values of 0.60 and 17.0  $\mu$ M for the uptake of [<sup>3</sup>H]5-HT and [<sup>3</sup>H]MPP<sup>+</sup>, respectively. Kinetic analysis of outward transport was performed by prelabeling the cells with increasing concentrations of the two substrates and exposing them to a saturating concentration of *p*-chloroamphetamine (PCA; 10  $\mu$ M). Apparent  $K_m$  values for PCA induced transport were 564  $\mu$ M and about 7 mM intracellular [<sup>3</sup>H]5-HT and [<sup>3</sup>H]MPP<sup>+</sup>, respectively. Lowering the extracellular Na<sup>+</sup> concentrations in uptake and superfusion experiments revealed

differential effects on substrate transport: at 10 mM Na<sup>+</sup> the  $K_m$  value for [<sup>3</sup>H]5-HT uptake increased ~5-fold and the  $V_{max}$  value remained unchanged. The  $K_m$  value for [<sup>3</sup>H]MPP<sup>+</sup> uptake also increased, but the  $V_{max}$  value was reduced by 50%. When efflux was studied at saturating prelabeling conditions of both substrates, PCA as well as unlabeled 5-HT and MPP<sup>+</sup> (all substances at saturating concentrations) induced the same efflux at 10 mM and 120 mM Na<sup>+</sup>. Thus, notwithstanding a 50% reduction in the  $V_{max}$  value of transport into the cell, MPP<sup>+</sup> was still able to induce maximal outward transport of either substrate. Thus, hSERT-mediated inward and outward transport seems to be independently modulated and may indicate inconsistencies with the classical model of facilitated exchange diffusion.

Synaptic clearance of serotonin [5-hydroxytryptamine (5-HT)] after release from nerve terminals is determined by the action of the plasma membrane serotonin transporter (SERT). SERT-mediated 5-HT uptake is driven by transmembrane ion gradients (Rudnick, 1997) and is blocked by a variety of drugs most prominently the tricyclic antidepressants (e.g., imipramine) and the selective serotonin uptake inhibitors (e.g., fluoxetine). As postulated by the theory of

facilitated exchange diffusion drugs causing 5-HT release are substrates of the transporter (e.g., the amphetamine derivatives such as *para*-chloroamphetamine, PCA), are taken up into the cells, and lead to an increase in the availability of inward-facing transporter binding sites for efflux of cytoplasmic 5-HT (Fuller et al., 1965; Fischer and Cho, 1979; Trendelenburg, 1989; Rudnick and Wall, 1992a,b; Gobbi et al., 1993; Rudnick, 1997). In this strict alternating access model, the rate of extracellular solute influx determines the rate of intracellular solute efflux. The faster the carrier can flip to the inside, the faster it can return to the outside carrying the effluxed substrate. In such a model one would expect influx and efflux rates to be modulated equivalently.

Although there is a wealth of data on the kinetic features of 5-HT inward transport in various experimental systems such as brain slices (Ross and Renyi, 1975), synaptosomes (Gobbi et al., 1993), platelets (Rudnick, 1977), and cells transfected with the SERT cDNA (Barker et al., 1994), infor-

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H.H.S. and P.S. contributed equally to this work.

**ABBREVIATIONS:** 5-HT, 5-hydroxytryptamine; SERT, serotonin transporter; PCA, *p*-chloroamphetamine; hSERT, human serotonin transporter; DAT, dopamine transporter; hNET, human norepinephrine transporter; RDEV, rotating disk electrode voltammetry; HEK, human embryonic kidney; MPP<sup>+</sup>, 1-methyl-4-phenylpyridinium; GFP, green fluorescent protein.

mation on outward transport is much more limited in this respect. Although cloning and heterologous expression of the human SERT (hSERT) in mammalian cells has made it possible to study the transporter function isolated from interfering factors such as vesicular storage or exocytotic release (Ramamoorthy et al., 1993), the acquirement of quantitative data on outward transport has remained difficult.

Important contributions on human dopamine and norepinephrine transporters (human DAT and hNET, respectively) have been provided by Justice's group using rotating disk electrode voltammetry (RDEV; Burnette et al., 1996; Chen et al., 1998, 1999; Chen and Justice, 1998). There are, nevertheless, no data on 5-HT transport obtained by this method. Moreover, RDEV, although providing excellent time resolution of transport in the second time frame, does not allow the change of media during the course of an experiment. Thus, the investigation of reverse transport caused by a change in the ionic composition of the medium is not possible. In the present experiments, we have used a superfusion system to study outward transport of [<sup>3</sup>H]5-HT from human embryonic kidney 293 cells (HEK 293 cells) expressing the hSERT. Two different labeled substrates were used to preload the cells: [<sup>3</sup>H]5-HT, the physiological substrate, which is able to diffuse through lipid bilayers easily relative to [<sup>3</sup>H]1-methyl-4-phenylpyridinium ([<sup>3</sup>H]MPP<sup>+</sup>), which is less lipophilic and therefore passes biological membranes to a smaller extent (Scholze et al., 2000). Release of radiolabel was initiated by adding transporter substrates to the medium (PCA, unlabeled 5-HT, or MPP<sup>+</sup>) or by lowering the extracellular Na<sup>+</sup> concentration, a measure known to favor reverse function of monoamine transporters (Liang and Rutledge, 1982; Bönisch, 1986; Chen et al., 1998; Piffl and Singer, 1999). The experiments allowed us to describe kinetic properties of hSERT-mediated outward transport in quantitative terms and to relate them to data on inward transport obtained in the same cells. The fact that different substrates were used for labeling the cells and that outward transport was triggered by different means revealed additional information regarding the molecular mechanisms of the translocation process of hSERT substrates. The present analyses demonstrate that at lowered extracellular Na<sup>+</sup> concentrations inward transport of MPP<sup>+</sup>, but not of 5-HT, is substantially reduced, whereas outward transport remains fully operative in cells preloaded with either substrate.

## Materials and Methods

### Cell Line Transfection

The cDNA for the human plasmalemmal serotonin transporter (Ramamoorthy et al., 1993) was a generous gift of Dr. R. D. Blakely (Department of Pharmacology and Center for Molecular Neuroscience, School of Medicine, Vanderbilt University, Nashville, TN). The coding region was subcloned into pEGFP-C1 (CLONTECH, Palo Alto, CA) removing the GFP coding region as described previously (Scholze et al., 2000; thus producing an hSERT lacking any GFP tag). For stable expression into HEK 293 cells the same method was used as described previously (Piffl et al., 1996). The stable transfectants (hSERT cells) were grown in minimal essential medium with Earle's salts and L-analyt-L-glutamine (L-glutamax I; Life Technologies, Grand Island, NY), 10% heat-inactivated fetal bovine serum, 50 mg/l gentamicin, and 500 μg/ml geneticin (G418) on 100-mm-diameter cell culture dishes at 37°C in an atmosphere of 5% CO<sub>2</sub>, 95% air.

### Uptake Experiments

The experiments were performed as described previously (Scholze et al., 2000). In brief,  $5 \times 10^4$  cells were seeded onto poly(D-lysine)-coated 24-well plates, and influx was measured 2 days after plating. Each well was washed twice with 1 ml of Krebs-Ringer-HEPES buffer (10 mM HEPES, 120 mM NaCl, 3 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 20 mM glucose, final pH 7.3). The cells were incubated with 0.2 μCi of [<sup>3</sup>H]5-HT (24.5 Ci/mmol) or [<sup>3</sup>H]MPP<sup>+</sup> (77.5 Ci/mmol), and various concentrations of unlabeled 5-HT or MPP<sup>+</sup> in a final volume of 0.2 ml. After the given incubation period at room temperature, the uptake buffer was aspirated rapidly and the cells were washed twice with 1 ml of ice-cold buffer. Cells were lysed with 0.5 ml of 1% SDS and transferred into scintillation vials for liquid scintillation counting. Nonspecific uptake was defined as uptake in the presence of 30 μM clomipramine and amounted to less than 2% of the total uptake. In some experiments low sodium buffers (0, 3, 10, 30, or 60 mM Na<sup>+</sup>) were used (NaCl was iso-osmotically replaced by choline chloride). The  $V_{\max}$  values for both substrates used in this study declined over time, indicating a reduction in the expression level during subsequent passages. The reason for this is not known; one possible explanation is a time-dependent increase of the amount of transporter being phosphorylated followed by internalization for recycling (Ramamoorthy and Blakely, 1999).

### Superfusion Experiments

Cells were grown overnight on round glass coverslips (5-mm diameter;  $4 \times 10^4$  cells/coverslip) incubated with [<sup>3</sup>H]5-HT or [<sup>3</sup>H]MPP<sup>+</sup> at different concentrations for 20 min at 37°C in a final volume of 0.225 ml of culture medium. Coverslips were then transferred to small superfusion chambers (0.2 ml) and superfused with Krebs-Ringer-HEPES buffer (25°C, 0.7 ml/min) as described previously (Piffl et al., 1995). After a washout period of 45 min to establish a stable efflux of radioactivity the experiment was started with the collection of fractions (4-min or 30-s duration). At the end of the experiment cells were lysed in 1% SDS. Tritium in the superfusate fractions and in the SDS-lysates was determined by liquid scintillation counting. High-performance liquid chromatography analysis revealed that >95% of the radioactivity in superfusates and cells coeluted with authentic [<sup>3</sup>H]5-HT (Scholze et al., 2000). Release of <sup>3</sup>H was expressed either quantitatively (pmol/min/10<sup>6</sup> cells or pmol/min) or as fractional rate; i.e., the radioactivity released during a fraction was expressed as percentage of the total radioactivity present in the cells at the beginning of that fraction.

**Determination of Cell Numbers per Coverslip.** For the estimation of the number of cells used in the superfusion experiments,  $4 \times 10^4$  cells were seeded onto round glass coverslips and grown overnight. Then the coverslips were removed from the cell culture wells, washed, immersed in 60 μl of 1% SDS, and the protein content of the cell lysate was measured (bicinchoninic acid kit; Pierce, Rockford, IL). Cell numbers were calculated using a standard curve generated on the same day from known amounts of the same cells. This assay was performed in six parallel incubations on five different days and yielded a mean cell number of  $27,165 \pm 2,822$ /coverslip ( $n = 30$ ). Furthermore, it was established in a separate series of experiments that there was no loss of cells during 1.5 h of superfusion (i.e., during the entire length of a superfusion experiment). For all calculations, a cell number of 27,000 cells/coverslip was used.

**Determination of the Cell Volume and Intracellular Substrate Concentration.** Cells ( $5 \times 10^6$ , this amounts to approximately 2 mg of cell protein) were suspended in 1 ml of Krebs-Ringer-HEPES buffer, containing 10 μCi [<sup>3</sup>H]H<sub>2</sub>O and 26.4 μCi [<sup>14</sup>C]inulin and incubated at 37°C for 20 min. The cell pellets were collected by centrifugation at 1000g for 2 min and lysed with 0.1% Triton X-100 (in 5 mM Tris-HCl; pH 7.4). The radioactivity of samples was measured by liquid scintillation counting. Intracellular water space was calculated as the difference between total [<sup>3</sup>H]H<sub>2</sub>O space and extracellular [<sup>14</sup>C]inulin space and amounted to  $1.25 \pm 0.13$  pl/cell

(mean  $\pm$  S.E. of three determination on three different days). Intracellular concentrations were calculated from the accumulated radioactivity in the cells, the above-mentioned value for cell volume and a cell number of 27,000 cells/coverlip.

## Chemicals

Tissue culture reagents were from Life Technologies. [ $^3\text{H}$ ]MPP $^+$  and [ $^3\text{H}$ ]5-HT were from PerkinElmer Life Sciences Products (Boston, MA). *para*-Chloroamphetamine, imipramine, and 5-HT were from Sigma-Aldrich Handels GmbH (Vienna, Austria) and MPP $^+$  was from RBI/Sigma, Natick, MA). All other chemicals were from commercial sources.

## Data Calculation

All curve fitting was done using Prism (GraphPad, San Diego, CA) nonlinear fitting, and plotting software. All results were expressed as mean  $\pm$  S.E.

## Results

### Uptake

Saturation analysis of [ $^3\text{H}$ ]5-HT or [ $^3\text{H}$ ]MPP $^+$  uptake in HEK 293 cells permanently expressing the hSERT (hSERT cells) revealed decreasing  $V_{\text{max}}$  values with increasing time in culture. Values for [ $^3\text{H}$ ]5-HT uptake were 1048 and 160 pmol/min/ $10^6$  cells after 3.5 and 11 weeks, respectively. Similarly,  $V_{\text{max}}$  values for [ $^3\text{H}$ ]MPP $^+$  uptake decreased from 1377 pmol/min/ $10^6$  cells after 3.5 weeks to 194 pmol/min/ $10^6$  cells after 7.5 weeks. In parallel uptake experiments performed using both substrates on the same day  $V_{\text{max}}$  values were consistently comparable ( $V_{\text{max}}$  [ $^3\text{H}$ ]5-HT/ $V_{\text{max}}$  [ $^3\text{H}$ ]MPP $^+$   $0.91 \pm 0.06$ ,  $n = 8$ , 95% CI, 0.79–1.02). The  $K_m$  values, however, were constant over time with  $0.60 \pm 0.07 \mu\text{M}$  (mean  $\pm$  S.E. of 22 independent determinations) and  $17.0 \pm 1.6 \mu\text{M}$  (mean  $\pm$  S.E. of 11 independent determinations) for [ $^3\text{H}$ ]5-HT and [ $^3\text{H}$ ]MPP $^+$ , respectively.

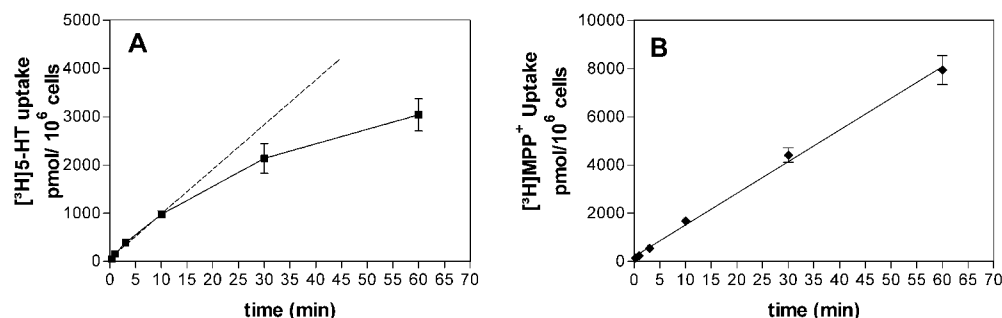
The time course of specific accumulation of substrates in hSERT cells after incubation with  $2.5 \mu\text{M}$  [ $^3\text{H}$ ]5-HT and  $75 \mu\text{M}$  [ $^3\text{H}$ ]MPP $^+$  is shown in Fig. 1. Although [ $^3\text{H}$ ]5-HT uptake was linear during the first 10 min only, uptake of [ $^3\text{H}$ ]MPP $^+$  was linear over the entire 60-min incubation period.

### Efflux

**Intracellular Substrate Concentration.** Efflux experiments were done by preincubating the cells with labeled substrates ( $37^\circ\text{C}$ , 20 min) followed by superfusion. After a

washout period of 45 min, which is necessary to establish a stable efflux of radioactivity, the collection of fractions was started. The relationship between the concentration of substrate during the preincubation period and the estimated intracellular substrate concentration at the beginning of fraction collection (i.e., after the washout) is shown in Fig. 2. Intracellular amounts of [ $^3\text{H}$ ]5-HT and [ $^3\text{H}$ ]MPP $^+$  displayed a concentration-dependent increase reaching saturation at preincubation concentrations of 5 and  $64 \mu\text{M}$ , respectively. The maximal intracellular concentrations reached depended on the time the cells had been kept in culture. For [ $^3\text{H}$ ]5-HT the values were around 2.5 and 1.2 mM after 3.5 and 8 weeks, respectively. For [ $^3\text{H}$ ]MPP $^+$  an 8-week value of 5 mM and a 12-week value of 3.5 mM were determined (values for shorter times in culture not available with [ $^3\text{H}$ ]MPP $^+$  efflux experiments).

To study the properties of reverse transport cells were exposed to a saturating concentration of the 5-HT-releasing drug PCA ( $10 \mu\text{M}$ ; Rudnick and Wall, 1992b; Scholze et al., 2000), and the time course of efflux was monitored in detail. The results are presented in Fig. 3. After the addition of PCA to superfused hSERT cells preincubated with  $5 \mu\text{M}$  [ $^3\text{H}$ ]5-HT or  $10 \mu\text{M}$  [ $^3\text{H}$ ]MPP $^+$  efflux velocities increased within 3 to 4 min, stayed constant for about 16 min, and subsequently decreased, indicating substrate depletion inside the cell. Maximal values were determined by averaging the data points between 8 and 16 min after drug addition. The effect of the uptake inhibitor imipramine ( $10 \mu\text{M}$ ) was investigated and analyzed in the same manner. Imipramine caused a noticeable increase in efflux of [ $^3\text{H}$ ]5-HT, an effect that is due to interruption of high affinity reuptake of amine leaving the cells by diffusion (Scholze et al., 2000). In all analyses of [ $^3\text{H}$ ]5-HT efflux, therefore, the effect of PCA was corrected for imipramine-induced efflux, which was determined in parallel superfusions. This correction was not necessary in [ $^3\text{H}$ ]MPP $^+$  experiments because this substrate is much less diffusible and does not undergo appreciable reuptake under superfusion conditions (no effect of imipramine in Fig. 3B; see also Scholze et al., 2000). In Fig. 3, C and D, the results are plotted as rates of fractional efflux; i.e., the radioactivity in each fraction was expressed as percentage of the total activity present in the cell at the beginning of that fraction. The fractional rates stayed constant at their maximal value over a considerably longer period of time than the corresponding velocities (up to 45 min after drug addition).



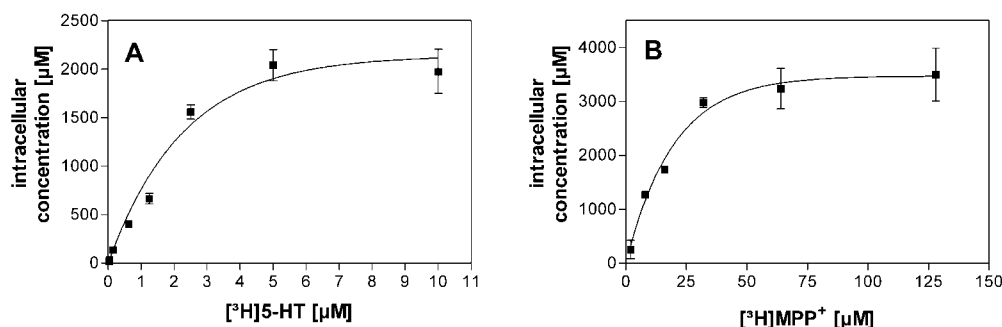
**Fig. 1.** Time course of [ $^3\text{H}$ ]5-HT and [ $^3\text{H}$ ]MPP $^+$  uptake in HEK 293 cells stably expressing the hSERT. HEK 293 cells stably expressing the hSERT were distributed in 24-well plates as described under *Materials and Methods* ( $5 \times 10^4$  cells) and incubated in the presence of  $2.5 \mu\text{M}$  [ $^3\text{H}$ ]5-HT (A) or  $75 \mu\text{M}$  [ $^3\text{H}$ ]MPP $^+$  (B) for different periods of time, washed, solubilized, and the tritium activity was determined by liquid scintillation counting. Nonspecific uptake was defined as uptake in the presence of  $30 \mu\text{M}$  clomipramine. Symbols represent mean  $\pm$  S.E. ( $n = 6$  from three separate experiments). Linear regression analysis was performed using the data points from 0 to 10 min in A (broken line) and all data points in B. Cells for experiments in A, 11 weeks in culture; cells for experiments in B, 7 weeks in culture.



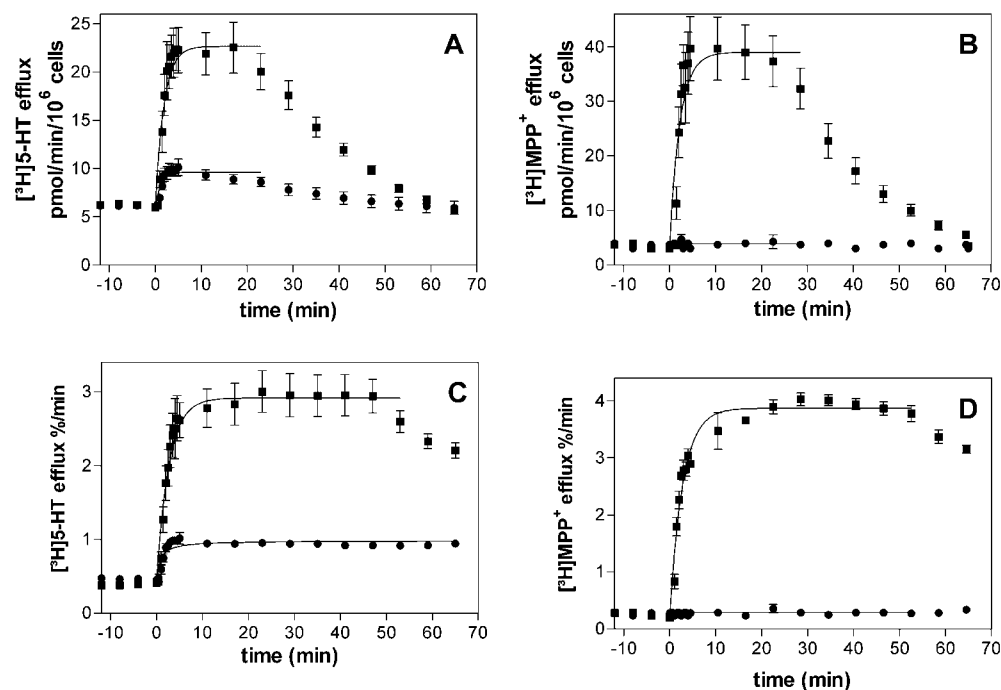
Figure 4 shows the results of two sets of experiments in which the relationship between intracellular substrate concentration and rates of efflux induced by PCA was analyzed. In [ $^3\text{H}$ ]5-HT experiments (Fig. 4A) the velocity of PCA (10  $\mu\text{M}$ )-induced outward transport (calculated as total efflux in the presence of PCA minus diffusion based efflux in the presence of imipramine) reached a well defined maximum. The intracellular concentration of [ $^3\text{H}$ ]5-HT at which the half-maximal rate of reverse transport was reached ( $K_m$  for reverse transport) was 564  $\mu\text{M}$ . In a second analysis (data not shown) the value was 323  $\mu\text{M}$ . In [ $^3\text{H}$ ]MPP $^+$  experiments (Fig. 4B) the velocity of outward transport also increased

with increasing intracellular substrate, but did not reach a maximum. Thus, it was not possible to calculate a half-maximal transport rate. An approximate estimate based on the nonlinear fit in Fig. 4B yielded a value of 7 mM and a maximal velocity for reverse transport of 158 pmol/min/ $10^6$  cells.

**Inward and Outward Transport Rates under Conditions of Reduced Extracellular Sodium.** Another series of experiments was done in which uptake initial rates of [ $^3\text{H}$ ]5-HT (100  $\mu\text{M}$ ) were measured at various  $\text{Na}^+$  concentrations. There was comparable accumulation of [ $^3\text{H}$ ]5-HT in all buffers containing 10 mM  $\text{Na}^+$  or more, whereas marked



**Fig. 2.** Intracellular retention of [ $^3\text{H}$ ]5-HT and [ $^3\text{H}$ ]MPP $^+$  in superfusion experiments. HEK 293 cells stably expressing the hSERT were grown on 5-mm glass coverslips, preincubated with various concentrations of [ $^3\text{H}$ ]5-HT (A) or [ $^3\text{H}$ ]MPP $^+$  (B), transferred to superfusion chambers, and superfused for 45 min at room temperature. The cells were then lysed, and the retained radioactivity determined and used for the calculation of intracellular concentrations. As outlined under *Materials and Methods*, the calculation was based on a cell volume of 1.25 pL/cell and a cell number of 27,000 cells/coverslip. Symbols represent mean  $\pm$  S.E. ( $n = 8$ –16 from at least two separate experiments; one observation is one superfusion chamber). Data were fitted using nonlinear regression analysis. Cells for experiments in A, 3.5 weeks in culture; cells for experiments in B, 12 weeks in culture.



**Fig. 3.** Determination of outward transport rates ( $V_{\text{efflux}}$ ). HEK 293 cells stably expressing the hSERT (4–5 weeks in culture) were grown on 5-mm coverslips, preincubated with [ $^3\text{H}$ ]5-HT (5  $\mu\text{M}$ ; A and C) or [ $^3\text{H}$ ]MPP $^+$  (64  $\mu\text{M}$ ; B and D) and superfused. After a 45-min washout period four 4-min fractions of basal efflux were collected. Then the buffer was switched to a buffer containing 10  $\mu\text{M}$  PCA (■) or 10  $\mu\text{M}$  imipramine (●), and ten 30-s fractions followed by ten 6-min fractions were collected. A and B, data are expressed as pmol/min/ $10^6$  cells calculated with a number of 27,000 cells/coverslip (under *Materials and Methods*). C and D, data of A and B, respectively, are shown as fractional rates; i.e., the radioactivity in each superfusate fraction was expressed as percentage of the activity in the cells at the beginning of that fraction. Efflux rates ( $V_{\text{efflux}}$ ) were estimated using nonlinear fitting of the individual data points from 0 min (time of drug addition) to 15 min (A and B) or 0 to 45 min (C and D). The mean value of the four baseline fractions was used for correction and subtracted from each data point before the fitting procedure was performed. Symbols represent mean  $\pm$  S.E. ( $n = 8$ –12, from at least two separate experiments; one observation is one superfusion chamber).

decreases to 44.2 and 10.5% of control values were observed at 3 and 0 mM Na<sup>+</sup>, respectively (Fig. 5A). Subsequently, saturation analyses for [<sup>3</sup>H]5-HT uptake at 10 and 120 mM Na<sup>+</sup> were performed. As shown for a representative experiment in Fig. 5B the  $K_m$  value for [<sup>3</sup>H]5-HT increased at 10 mM Na<sup>+</sup>, whereas the  $V_{max}$  value did not change. Mean  $K_m$  values across all analyses increased from  $0.6 \pm 0.07 \mu\text{M}$  ( $n = 22$ ) under 120 mM Na<sup>+</sup> to  $3.29 \pm 0.41 \mu\text{M}$  ( $n = 8$ ) under 10 mM Na<sup>+</sup>.

In parallel to the uptake experiments  $V_{efflux}$  values for PCA induced [<sup>3</sup>H]5-HT release at reduced concentrations of Na<sup>+</sup> were determined. Representatively, the time course of the 10 mM Na<sup>+</sup> experiments is shown in Fig. 5C. The cells were preincubated with 5  $\mu\text{M}$  [<sup>3</sup>H]5-HT to obtain the maximally achievable intracellular substrate concentration. Superfusion was started at 120 mM Na<sup>+</sup>, and then switched to 10 mM Na<sup>+</sup> followed by addition of 10  $\mu\text{M}$  PCA. Controls were exposed to PCA but not to 10 mM Na<sup>+</sup>. As expected, lowering of Na<sup>+</sup> caused a distinct increase in efflux, which reached its maximum within 8 min. The addition of PCA resulted in a further increase, which, under this experimental condition, attained the same level as PCA-induced efflux at control conditions (120 mM Na<sup>+</sup>). The effects of the various low-sodium buffers on the efflux of [<sup>3</sup>H]5-HT and the action of PCA are summarized in Fig. 5D (open columns). Reduction of Na<sup>+</sup> caused increases in efflux of [<sup>3</sup>H]5-HT, starting at 60 mM Na<sup>+</sup> with 6.2% of control and reaching a maximum at 0 Na<sup>+</sup> with 48% of control (control is efflux induced by 10  $\mu\text{M}$  PCA at 120 mM Na<sup>+</sup>). The addition of PCA did not cause any further increase in efflux at 0 and 3 mM Na<sup>+</sup>, but exerted its full effect at concentrations of >10 mM Na<sup>+</sup> (Fig. 5D, closed columns).

Experiments on uptake and PCA-induced efflux at 10 mM sodium were also performed using [<sup>3</sup>H]MPP<sup>+</sup> as substrate (64  $\mu\text{M}$ ). In the presence of 10 mM Na<sup>+</sup> the  $K_m$  value for [<sup>3</sup>H]MPP<sup>+</sup> uptake increased (from 49 to 264  $\mu\text{M}$ ; Fig. 6A), but, in contrast to the results with [<sup>3</sup>H]5-HT, the  $V_{max}$  value was greatly diminished (from 830 to 320 pmol/min/10<sup>6</sup> cells; Fig. 6A). However, the exposure to 10 mM Na<sup>+</sup> did not

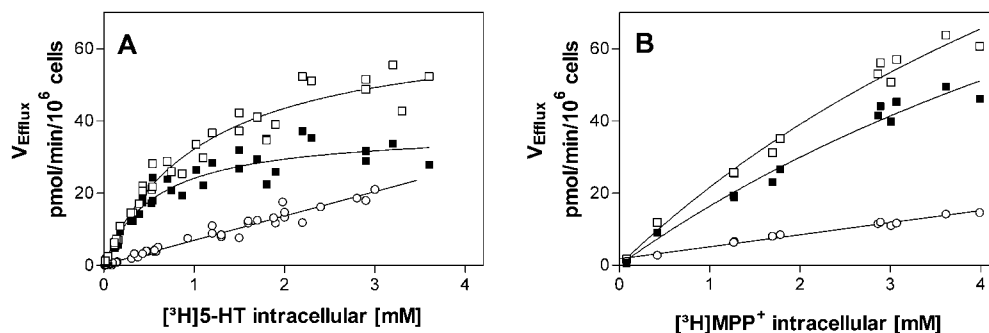
compromise PCA-induced efflux in the superfusion experiment (Fig. 6B).

This was also shown by determining maximal initial uptake rates and maximal rates of PCA-induced efflux at 120 and 10 mM Na<sup>+</sup> for both [<sup>3</sup>H]5-HT and [<sup>3</sup>H]MPP<sup>+</sup> in batches of cells that had been kept in culture for different periods of time (Fig. 7). Saturation analyses were performed to obtain  $V_{max}$  values, and  $V_{efflux}$  values were determined under conditions of the maximally obtainable intracellular substrate concentration (i.e., after preincubation with 5  $\mu\text{M}$  [<sup>3</sup>H]5-HT and 64  $\mu\text{M}$  [<sup>3</sup>H]MPP<sup>+</sup>). There were highly significant linear relationships between  $V_{max}$  and  $V_{efflux}$  values for each substrate (120 mM Na<sup>+</sup>, [<sup>3</sup>H]5-HT:  $r^2 = 0.946$ ; 120 mM Na<sup>+</sup>, [<sup>3</sup>H]MPP<sup>+</sup>:  $r^2 = 0.943$ ; 10 mM Na<sup>+</sup>, [<sup>3</sup>H]5-HT:  $r^2 = 0.813$ ; and 10 mM Na<sup>+</sup>, [<sup>3</sup>H]MPP<sup>+</sup>:  $r^2 = 0.966$ ). With regard to the two Na<sup>+</sup> concentrations, the slopes of the regression lines were superimposable for [<sup>3</sup>H]5-HT (Fig. 7A) but clearly distinct for [<sup>3</sup>H]MPP<sup>+</sup> (Fig. 7B), indicating that low Na<sup>+</sup> compromised outward transport less than inward transport.

To study transporter-mediated efflux induced by substrates with well characterized transport and diffusion properties a further set of experiments was performed using unlabeled 5-HT and MPP<sup>+</sup> instead of PCA. After loading with 5  $\mu\text{M}$  [<sup>3</sup>H]5-HT or 64  $\mu\text{M}$  [<sup>3</sup>H]MPP<sup>+</sup> cells were superfused and challenged with saturating concentrations of 5-HT (10  $\mu\text{M}$ ) and MPP<sup>+</sup> (1 mM) at 10 and 120 mM Na<sup>+</sup> (Fig. 8, A and B). For each substrate similar maximal efflux rates were achieved under all experimental conditions (i.e., irrespective of the substrate used for challenging or the prevailing buffer condition).

## Discussion

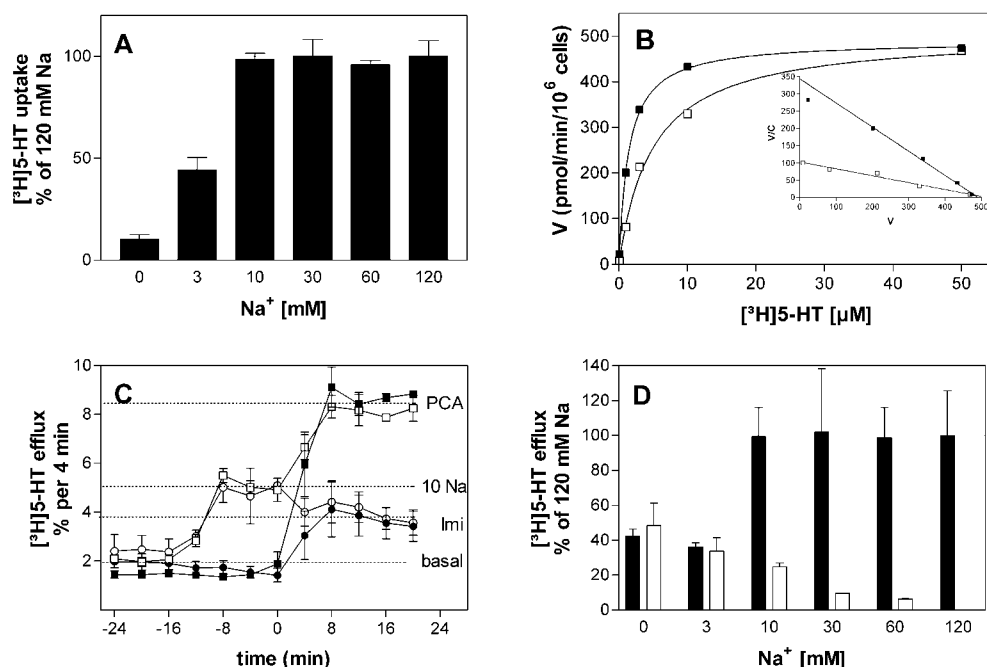
The aim of the present study was to analyze and to compare quantitative aspects of inward and outward transport of substrates by the human SERT to obtain more insight into the mechanisms of carrier-mediated release. As in our previous study on the rat (Sitte et al., 2000) and human SERT (Scholze et al., 2000), we used the natural substrate 5-HT,



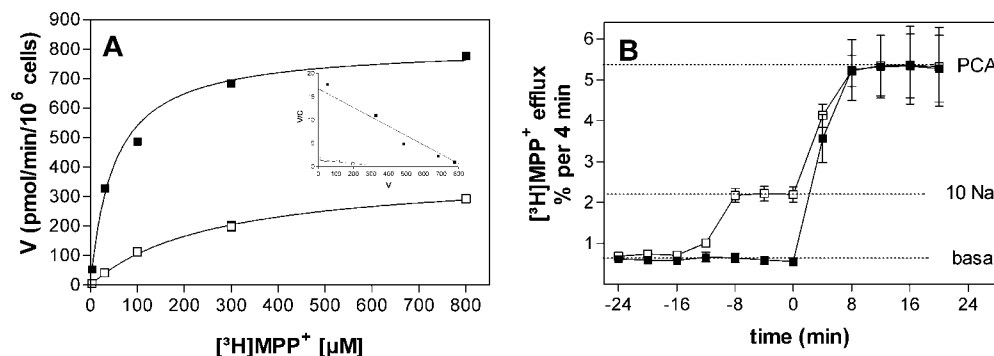
**Fig. 4.** Relationship between intracellular substrate concentration and PCA-induced efflux. HEK 293 cells stably expressing the hSERT were grown on 5-mm coverslips, preincubated with different concentrations of [<sup>3</sup>H]5-HT (0.15–10  $\mu\text{M}$ ) or [<sup>3</sup>H]MPP<sup>+</sup> (2–128  $\mu\text{M}$ ), superfused, challenged with 10  $\mu\text{M}$  PCA, and  $V_{efflux}$  values determined as explained in Fig. 3. In cells preincubated with [<sup>3</sup>H]5-HT  $V_{efflux}$  values for 10  $\mu\text{M}$  imipramine were also determined in parallel superfusion. Intracellular substrate concentrations at the time of drug addition were calculated from the sum of radioactivity remaining in the cells at the end of the experiment and the superfusate fractions. The calculation was based on a cell volume of 1.25 pL/cell and a cell number of 27,000 cells/coverslip (under *Materials and Methods*). A, [<sup>3</sup>H]5-HT experiments;  $V_{efflux}$  values for PCA (■) were calculated from total PCA-induced efflux (□) minus diffusion-based efflux estimated from the linear regression line of the  $V_{efflux}$  values for imipramine (○). Nonlinear fitting was used to calculate the maximal efflux rate ( $V_{max}$  for reverse transport; 38 pmol/min/10<sup>6</sup> cells) and that intracellular concentration of [<sup>3</sup>H]5-HT at which the half-maximal rate of reverse transport was reached ( $K_m$  for reverse transport; 564  $\mu\text{M}$ ; see also text). For each drug,  $n = 30$ ; one data point is one superfusion chamber. B, [<sup>3</sup>H]MPP<sup>+</sup> experiments;  $V_{efflux}$  values for PCA (■) were calculated from total PCA-induced efflux (□) basal efflux during the first 16 min before the addition of PCA (○). Nonlinear fitting was performed as in A. For calculated parameters ( $V_{max}$  and  $K_m$  for reverse transport), see text ( $n = 12$ ; one data point is one superfusion chamber). Cells for experiments in A, 4 weeks in culture; cells for experiments in B, 14 weeks in culture.

and MPP<sup>+</sup>, a commonly used substrate for all monoamine transporters (Wall et al., 1995). 5-HT and MPP<sup>+</sup> differ in their  $K_m$  value for the hSERT by a factor of about 25 (0.6 versus 17  $\mu$ M, respectively) and in their lipophilicity. These differences are the cause of some typical findings, which have been partly discussed previously (Scholze et al., 2000; Sitte et al., 2000) and will be reviewed here because of their relevance for the present findings.

Because the cells used in the present experiments do not possess a vesicular storage mechanism, accumulated 5-HT will leave the cytoplasm by diffusion, but is partly subject to reuptake because of its high affinity for the hSERT. A reuptake inhibitor such as imipramine causes a distinct increase in efflux, thus revealing the total nontransporter-mediated traffic of 5-HT out of the cell



**Fig. 5.** Inward and outward transport of [<sup>3</sup>H]5-HT under conditions of reduced extracellular sodium. A, HEK 293 cells stably expressing the hSERT were incubated in the presence of 100  $\mu$ M [<sup>3</sup>H]5-HT for 1 min in buffers containing different concentrations of Na<sup>+</sup> (Na<sup>+</sup> replaced by choline). For further experimental details, see legend to Fig. 1. Results are expressed as percentage of [<sup>3</sup>H]5-HT uptake at 120 mM Na<sup>+</sup> and represent mean  $\pm$  S.E. ( $n = 6$  from three separate experiments). B, saturation analysis. Cells were incubated in the presence of different concentrations of [<sup>3</sup>H]5-HT for 1 min in a buffer containing 120 mM Na<sup>+</sup> (■) or 10 mM Na<sup>+</sup> (□). For further experimental details, see legend to Fig. 1. Nonlinear fitting was used to calculate  $V_{max}$  and  $K_m$ . Inset displays Eadie-Hofstee plot of the same data. One of three experiments performed in duplicate is shown. C, superfusion experiment; cells were loaded with 5  $\mu$ M [<sup>3</sup>H]5-HT, superfused, and 4-min fractions collected. After three fractions (−16 min) the buffer was switched to a buffer containing 10 mM Na<sup>+</sup> (○, □; Na<sup>+</sup> replaced by choline) followed by the addition (0 min) of 10  $\mu$ M PCA (□) or 10  $\mu$ M imipramine (○). Controls (●, ■) received the same drugs but were not switched to 10 mM Na<sup>+</sup>. Dotted lines indicate mean fractional rates of efflux at the different experimental conditions. Symbols represent mean  $\pm$  S.E. ( $n = 9$  from three separate experiments). D, superfusion experiments as described in C were performed at different concentrations of extracellular Na<sup>+</sup>. Open columns represent efflux induced by low Na<sup>+</sup>, and closed columns represent efflux in the presence of low Na<sup>+</sup> plus PCA. All PCA effects were corrected for the efflux induced by imipramine at the corresponding buffer condition. All data are presented as percentage of the PCA effect at 120 mM Na<sup>+</sup>. Columns represent mean  $\pm$  S.E. ( $n = 6$  from two separate experiments). Experiments shown in B and C were performed on the same day. Cells for all experiments, 6 weeks in culture.



**Fig. 6.** Inward and outward transport of [<sup>3</sup>H]MPP<sup>+</sup> under conditions of reduced extracellular sodium. A, HEK 293 cells stably expressing the hSERT were incubated in the presence of different concentrations of [<sup>3</sup>H]MPP<sup>+</sup> for 1 min in a buffer containing 120 mM Na<sup>+</sup> (■) or 10 mM Na<sup>+</sup> (□). For further experimental details, see legend to Fig. 1. Nonlinear fitting was used to calculate  $V_{max}$  and  $K_m$ . Inset displays Eadie-Hofstee plot of the same data. One of three experiments performed in duplicate is shown. B, time course of superfusion experiments; cells were loaded with 64  $\mu$ M [<sup>3</sup>H]MPP<sup>+</sup>, superfused, and 4-min fractions collected. After three fractions (−16 min) the buffer was switched to a buffer containing 10 mM Na<sup>+</sup> (□; Na<sup>+</sup> replaced by choline) followed by the addition (0 min) of 10  $\mu$ M PCA (□). Controls (■) also received PCA but were not switched to 10 mM Na<sup>+</sup>. Dotted lines indicate mean fractional rates of efflux at the different experimental conditions. Symbols represent mean  $\pm$  S.E. ( $n = 9$  from three separate experiments). Experiments shown in A and B were performed on the same day. Cells for all experiments, 5 weeks in culture.

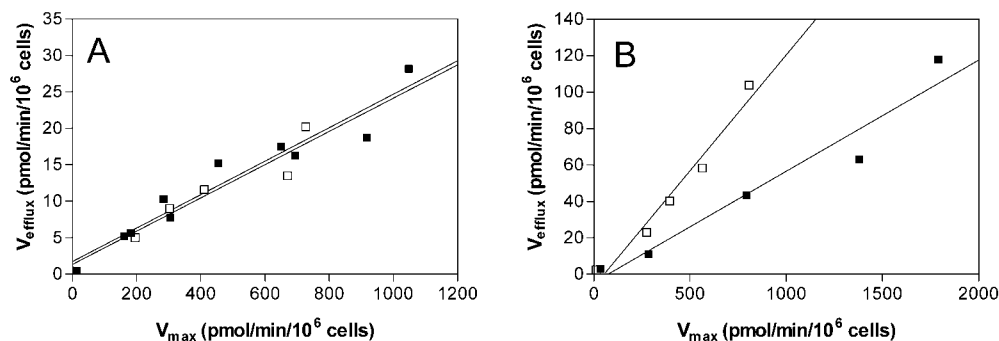
(Fig. 3A; note the linear and nonsaturable relationship between intracellular [ $^3\text{H}$ ]5-HT concentration and efflux rate in Fig. 4A). A similar observation was made by Chen et al. (1998) regarding [ $^3\text{H}$ ]dopamine efflux and the effect of cocaine in LLC-PK1 cells expressing the hNET. Therefore, transporter-mediated efflux of [ $^3\text{H}$ ]5-HT was always calculated as total efflux minus imipramine-induced efflux. By contrast, reuptake of  $\text{MPP}^+$  does not take place due to its low  $K_m$  value for the hSERT. Imipramine therefore has no effect on [ $^3\text{H}$ ]MPP $^+$  efflux (Fig. 3B), and the observed basal efflux equals total nontransporter-mediated efflux.

For the calculation of intracellular substrate concentrations the cell volume was determined as intracellular water space, which amounted to 1.25 pL/cell. This converts to 5.25  $\mu\text{L}/\text{mg}$  protein, which is in reasonable agreement with the value of 6.7  $\mu\text{L}/\text{mg}$  published by Schömig for the same cells (Martel et al., 1996) or the value of 3  $\mu\text{L}/\text{mg}$  published by Chen et al. (1998) for LLC-PK1 cells.

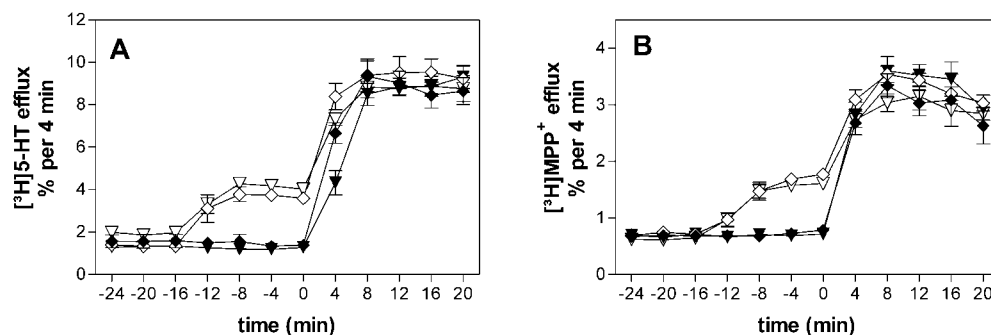
Based on these estimates, the cells apparently accumulated substrates up to millimolar concentrations. This is in the range of the value found by and Justice (1998b) for dopamine (0.52 mM). Reverse transport was initiated by addition of a saturating concentration of the 5-HT-releasing drug PCA (Rudnick and Wall, 1992b; Scholze et al., 2000). There was an initial acceleration period in efflux lasting about 5 min followed by a plateau and subsequent substrate depletion (Fig. 3). The time of 5 min to reach the maximal efflux level was longer than the time of <2 min for *m*-

tyramine-induced dopamine efflux observed by Chen and Justice (1998a) using RDEV in hNET expressing cells. The 2-fold difference may partly be due to the fact that our superfusion experiments are carried out at 25°C, whereas RDEV is performed at 37°C, but inherent differences between hNET and hSERT are also possible. There are at present no efflux data in hSERT expressing cells that could be used for comparison. The relationship between the estimated intracellular concentration of 5-HT and rates of 5-HT efflux during the stable phase of PCA-evoked efflux displayed saturation kinetics (Fig. 4A) with an apparent  $K_m$  value of 500- to 1000-fold the value found for inward transport. Assuming a similar difference for MPP $^+$ , an apparent  $K_m$  value in the millimolar range would be expected. It was not possible to achieve high enough intracellular concentrations experimentally to perform a correct analysis but a fit using the available data points rendered an estimate in support of this hypothesis (Fig. 4B).

This marked difference in the  $K_m$  value for inward and outward transport may be caused by several factors one of which is the low intracellular  $\text{Na}^+$  concentration. It is well known that the  $K_m$  value for substrate transport by monoamine transporters (Graefe and Bönisch, 1989) increases at low  $\text{Na}^+$ . In the present study, the  $K_m$  value for the inward transport of 5-HT and MPP $^+$  were about 5-fold higher at 10 mM  $\text{Na}^+$  compared with 120 mM  $\text{Na}^+$  (Figs. 5B and 6A), which is much less than the at least 500-fold difference between the  $K_m$  values for influx and efflux. Another factor



**Fig. 7.** Relationship between maximal rates of inward and outward transport of [ $^3\text{H}$ ]5-HT and [ $^3\text{H}$ ]MPP $^+$  under control conditions and under conditions of reduced extracellular sodium. Batches of cells that had been kept in culture for different periods of time (3.5–12 weeks) were used for uptake and superfusion experiments performed in parallel under standard conditions (■, 120 mM  $\text{Na}^+$ ) and low- $\text{Na}^+$  conditions (□, 10 mM;  $\text{Na}^+$  replaced by choline).  $V_{\text{max}}$  values for the uptake of [ $^3\text{H}$ ]5-HT and [ $^3\text{H}$ ]MPP $^+$  were determined using saturation analyses as described in legends to Figs. 5B and 6A, respectively.  $V_{\text{efflux}}$  values were determined in superfused cells preincubated with 5  $\mu\text{M}$  [ $^3\text{H}$ ]5-HT or 64  $\mu\text{M}$  [ $^3\text{H}$ ]MPP $^+$  and challenged with 10  $\mu\text{M}$  PCA. For experimental details, see legend to Fig. 3. A, [ $^3\text{H}$ ]5-HT experiments; B, [ $^3\text{H}$ ]MPP $^+$  experiments. For correlation coefficients see text.



**Fig. 8.** [ $^3\text{H}$ ]5-HT and [ $^3\text{H}$ ]MPP $^+$  efflux induced by 5-HT and MPP $^+$  under standard and low-sodium conditions. HEK 293 cells stably expressing the hSERT were loaded with 5  $\mu\text{M}$  [ $^3\text{H}$ ]5-HT (A, cells 5 weeks in culture) or 64  $\mu\text{M}$  [ $^3\text{H}$ ]MPP $^+$  (B, cells 3.5 weeks in culture), superfused, and 4-min fractions collected. After three fractions (–16 min) the buffer was switched to a buffer containing 10 mM  $\text{Na}^+$  ( $\text{Na}^+$  replaced by choline) followed by the addition (0 min) of 10  $\mu\text{M}$  5-HT (◇) or 1 mM MPP $^+$  (▽). Controls (▼, ◆) received the same drugs but were not switched to 10 mM  $\text{Na}^+$ .



may be competition of substrates inside the cell. It would be conceivable that PCA, in addition to being taken up, also diffuses into the cell and reaches concentrations high enough to compete with intracellular substrate for outward transport. It is noteworthy, however, that a very similar difference in  $K_m$  values for in- and outward transport of dopamine was observed in hNET expressing cells (0.88 versus 396  $\mu\text{M}$ ; Chen and Justice, 1998).

The  $V_{\text{max}}$  values for both substrates used in this study depended on the time the cells had been kept in culture, as also observed by others recently (Qian et al., 1997; Ramamoorthy and Blakely, 1999). However, uptake velocities were comparable for both substrates when cells with the same time in culture were used in parallel assays. The  $V_{\text{max}}$  value for substrate uptake and the maximal velocity of PCA-induced substrate efflux ( $V_{\text{efflux}}$ ) displayed a constant relationship independent of time in culture;  $V_{\text{max}}/V_{\text{efflux}}$  ratios were 40 and 20 for 5-HT and MPP<sup>+</sup>, respectively (Fig. 7). The fact that these ratios are not the same for the two substrates indicates that the hSERT handles substrates differently with regard to transport direction. This, in turn, may be explained by a differential influence of the low intracellular sodium concentration on binding and transport of 5-HT and MPP<sup>+</sup>. With this possibility in mind, an investigation of the sodium dependence of 5-HT transport was performed (Fig. 5). Lowering the extracellular Na<sup>+</sup> concentration to 10 mM did not change the  $V_{\text{max}}$  value of 5-HT uptake (albeit the  $K_m$  value was markedly increased) and also did not influence  $V_{\text{efflux}}$ . Therefore,  $V_{\text{max}}/V_{\text{efflux}}$  ratios for 5-HT were the same at 10 and 120 mM Na<sup>+</sup> (Fig. 7A). The same reduction of Na<sup>+</sup> in MPP<sup>+</sup> experiments, however, resulted in a pronounced decline in the  $V_{\text{max}}$  value but no change in  $V_{\text{efflux}}$ . This reduced the  $V_{\text{max}}/V_{\text{efflux}}$  ratios for MPP<sup>+</sup> from 20 at 120 mM Na<sup>+</sup> to 10 at 10 mM Na<sup>+</sup> (Fig. 7B). The result clearly indicates that, at least in the case of MPP<sup>+</sup>, inward and outward transport by the hSERT can be independently modulated. This conclusion is further corroborated by the results of the experiments shown in Fig. 8. When superfusion assays at 120 and 10 mM Na<sup>+</sup> were performed using 5-HT and MPP<sup>+</sup> for induction of reverse transport, there were no differences in substrate induced efflux, although the  $V_{\text{max}}$  value of MPP<sup>+</sup> at 10 mM Na<sup>+</sup> is only half of that at 120 mM Na<sup>+</sup>. Thus, reverse transport rates did not seem to be strictly coupled to ratios of inward transport, irrespective whether the cells were loaded with [<sup>3</sup>H]5-HT or [<sup>3</sup>H]MPP<sup>+</sup>.

The results of our experiments with lowered extracellular Na<sup>+</sup> suggest that a conformational change of the hSERT can be induced that is unfavorable for inward transport but does not affect outward transport. The data may be interpreted in consonance with our previously formulated hypothesis that releasing substrates may not only be taken up but may also cause an influx of sodium ions and thus enhance the possibility of reverse transport (Sitte et al., 1998; Pifl and Singer, 1999) in agreement with a proposed channel mode of the transporter protein (Sonders and Amara, 1996; DeFelice and Blakely, 1996; Beckman and Quick, 1998). The hypothesis has received strong support by recent work of Galli et al. (2000) who demonstrated in HEK 293 cells expressing the hDAT that 1) amphetamine causes an inward current depolarizing the membrane, and 2) that intracellular Na<sup>+</sup> is required for amphetamine to commence dopamine efflux. There are also earlier reports showing requirement for inter-

nal Na<sup>+</sup> and Cl<sup>-</sup> for exchange (Rudnick and Wall, 1992a,b) and efflux (Nelson and Rudnick, 1979, 1982). However, given the many possible factors that are likely to determine the probability of efflux on any given cycle of the transporter (e.g., relative rates of conformational changes for influx and efflux, the internal Na<sup>+</sup>, Cl<sup>-</sup>, and K<sup>+</sup> concentrations and membrane potential), alternative explanations are possible. Thus, efflux may saturate under conditions where the rate of influx is not maximal (i.e., efflux is saturated above a threshold of influx).

A native backdrop for independent modulation of carrier-mediated inward and outward transport at the DAT may be provided by the results of Gnegy's group who have accumulated evidence that PKC activation may shift the activity of DATs from supporting inward transport exclusively to an enhanced tendency for amphetamine-induced efflux (Browman et al., 1998; Kantor and Gnegy, 1998). Finally, Chen and Justice (2000), using site-directed mutagenesis at the DAT, have revealed that a single mutation can affect only one direction of transport in bidirectional transport assays (Chen and Justice, 2000).

In conclusion, the present data provide for the first time information on quantitative aspects of hSERT-mediated reverse transport of substrates. Moreover, they highlight certain features of reverse transport that cannot be reconciled with the classical model of facilitated exchange diffusion.

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