Reconstitution of the human placental 5-hydroxytryptamine transporter in a catalytically active form after detergent solubilization

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The 5-hydroxytryptamine (5-HT; serotonin) transporter was solubilized from purified human placental brush border membranes by cholate in the presence of urea, and the solubilized transporter was reconstituted into proteoliposomes in a functionally active form. Solubilization of the membranes with cholate in the absence of urea inactivated the transporter. The reconstitution procedure involved precipitation of the solubilized proteins and simultaneous removal of cholate and urea by poly(ethylene glycol), and incorporation of the precipitated proteins into proteoliposomes in the presence of asolectin by a freeze-thaw/sonication technique. Optimal conditions included the use of 6% poly(ethylene glycol) during the precipitation step and an asolectin/protein ratio of 10:1 during the reconstitution step. K^+ was present in the reconstitution medium. The reconstituted proteoliposomes showed the ability to transport 5-HT against a concentration gradient when an inwardly directed NaCl gradient was imposed. The 5-HT transport system in the proteoliposomes had an absolute requirement for Na^+ and Cl^- . The system was specific for 5-HT and was inhibited by imipramine, paroxetine and fluoxetine. The $Na^+/Cl^-/5-HT$ stoichiometry was found to be 1:1:1. The transport process was electrically silent, indicating that one K^+ ion was countertransported for each 5-HT molecule. The reconstituted 5-HT transporter showed high affinity for 5-HT, with an apparent Michaelis-Menten constant of $0.34 \pm 0.01 \mu M$. It is concluded that the human placental 5-HT transporter can be solubilized and reconstituted into proteoliposomes in a transport-competent form and that the characteristics of the reconstituted transporter are similar to those of the native transporter.

INTRODUCTION

The plasma membrane 5-hydroxytryptamine (5-HT; serotonin) transporter present in the synaptosomes plays an important role in the regulation of serotonergic transmission [1–3]. This transporter is also present in the plasma membrane of platelets, where it participates in the uptake of circulating 5-HT for subsequent storage [4]. Numerous studies with isolated plasma membrane vesicles from brain and platelets have shown that the 5-HT transporter is energized by a transmembrane NaCl gradient (for reviews, see [1] and [3]). In addition to Na^+ and Cl^- , the system also interacts with H^+ and/or K^+ [5–9]. Many tricyclic (e.g. imipramine) and non-tricyclic (e.g. paroxetine) antidepressants interact with the 5-HT transporter with high affinity [10]. Because these antidepressants are non-transportable binding ligands for the transporter, they have been highly useful in the characterization of the transporter. We have previously reported that the brush border membranes isolated from term human placentas possess an active 5-HT transport mechanism [11]. Cultured cell lines derived from human placental choriocarcinoma also express this 5-HT transporter [12]. The human placental 5-HT transporter is very similar to the 5-HT transporter of the brain and the platelet in many characteristics, such as energization by a NaCl gradient [11–14], interaction with H^+ and K^+ [14], and inhibition by tricyclic and non-tricyclic antidepressants [15,16]. The physiological role of the 5-HT transporter in the function of the placenta and/or in the growth and development of the fetus is not known.

Many investigators have attempted to solubilize the transporter from either neuronal or platelet plasma membranes [17–23]. In all of these studies, without any exception, only the

binding of specific ligands such as imipramine or paroxetine was used to detect and quantify the 5-HT transporter in the solubilized material. The transport function was never measured. In order to assay the transport function, the solubilized transporter should be reconstituted into artificial membrane vesicles. Reconstitution of the solubilized proteins into proteoliposomes and demonstration of the transport function in these proteoliposomes will be necessary to prove unequivocally the identity of the solubilized protein. However, digitonin, which has been shown in many studies to be the most suitable detergent for solubilization of the 5-HT transporter, as monitored by assaying either imipramine or paroxetine binding, is unfortunately one of the least suitable detergents for reconstitution studies. Because of the very low critical micellar concentration of digitonin (0.01–0.04%), it is very difficult to remove this detergent by the routinely used methods such as dialysis or gel permeation chromatography during reconstitution.

Cholate has been widely used to solubilize a wide variety of transport proteins. Due to its relatively high critical micellar concentration (0.57%), cholate can be easily removed and therefore this detergent is more suitable than digitonin for reconstitution studies. However, there are conflicting claims on the ability of cholate to solubilize the 5-HT transporter [17,21,24]. Furthermore, even in those studies in which it was shown that cholate was able to solubilize the transporter and that the solubilized transporter could be reconstituted into proteoliposomes [21], the transporter function in the proteoliposomes was measured only by the binding of imipramine. Whether these proteoliposomes possessed the NaCl-gradient-dependent 5-HT transport activity was not examined.

Thus, even though numerous reports have described the

Abbreviations used: 5-HT, 5-hydroxytryptamine (serotonin); PEG, poly(ethylene glycol); PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol.

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successful solubilization of the plasma membrane 5-HT transporter, to our knowledge there has been no demonstration of reconstitution of the solubilized material into proteoliposomes which exhibit the NaCl-dependent 5-HT transport function. We report in this paper for the first time that the 5-HT transporter can be solubilized from human placental brush border membranes by cholate in the presence of urea, and that the solubilized transporter can be reconstituted into proteoliposomes in a transport-competent form.

EXPERIMENTAL

Materials

Cholate (sodium salt), poly(ethylene glycol) (PEG, average M_r 8000), phenylmethanesulphonyl fluoride (PMSF), valinomycin, imipramine, reserpine, 5-hydroxytryptophan, 5-HT, dopamine and noradrenaline were purchased from Sigma (St. Louis, MO, U.S.A.). Ultrapure urea was purchased from Pierce (Rockford, IL, U.S.A.). Asolectin was from Associated Concentrates (Woodside, NY, U.S.A.). Paroxetine and fluoxetine were gifts from Beecham Pharmaceuticals (Betchworth, Surrey, U.K.) and Eli Lilly Co. (Indianapolis, IN, U.S.A.) respectively. All other chemicals were of analytical grade.

5-[1,2- $^3\text{H}(\text{N})$]Hydroxytryptamine binoxalate (sp. radioactivity 30.4 Ci/mmol) and [2- $^3\text{H}(\text{N})$]taurine (sp. radioactivity 25.6 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA, U.S.A.).

Preparation of brush border membranes from human term placentas

Brush border membranes from normal human term placentas were prepared by an Mg^{2+} -aggregation method previously described from this laboratory [25,26]. In the final step, membranes were suspended in 5 mM-Hepes/Tris buffer, pH 7.4, containing 150 mM-NaCl. The protein concentration of the membrane suspension was adjusted to 10 mg/ml, and the suspension was stored in 1 ml aliquots in liquid N_2 until use.

Solubilization and reconstitution

The procedure employed in this study for solubilization of placental brush border membranes and for reconstitution of the solubilized proteins into proteoliposomes was a modification of the methods reported by McCormick *et al.* [27] and by Tamarappoo & Kilberg [28]. In this procedure, membranes were solubilized by cholate in the presence of urea and the solubilized proteins were precipitated with PEG and incorporated into proteoliposomes using asolectin in the presence of K^+ .

A 2 ml portion of the brush border membrane suspension (20 mg of protein) was thawed at 37 °C and mixed with 8 ml of solubilization buffer [10 mM-Hepes/Tris, pH 7.5, with 100 mM-NaCl, 0.1 mM-EDTA, 0.3 mM-dithiothreitol (DTT) and 1 μM -PMSF] containing 5 M-urea and 3.125% (w/v) cholate. The mixture was stirred for 30 min at 4 °C. During this solubilization step, the protein concentration was 2 mg/ml. The suspension was then diluted with an equal volume of solubilization buffer and centrifuged at 220 000 g for 1 h. The solubilized proteins were recovered in the clear supernatant. Without the dilution step, centrifugation of the original suspension under identical conditions failed to yield a clear supernatant. The insoluble material recovered in the pellet was also saved in some experiments and suspended in solubilization buffer containing 1.25% cholate and 2 M-urea. Proteins in the supernatant and in the pellet suspension were precipitated with PEG. Stock solutions of different concentrations of PEG were made in the solubilization buffer, and 2 vol. of the supernatant were mixed

with 1 vol. of PEG solution to give the desired concentration of PEG. In most experiments a 18% PEG solution was used, which gave a final PEG concentration of 6%. The mixture was kept on ice for 15 min and then centrifuged at 140 000 g for 30 min. The resulting pellet was rinsed four times with the reconstitution buffer (10 mM-Hepes/Tris, pH 7.5, 200 mM-potassium gluconate and 1 mM-magnesium gluconate) and then suspended in a small volume of the same buffer by passing through a 25 gauge needle. The protein concentration of the suspension was adjusted to 1 mg/ml and the suspension was immediately used for reconstitution. In some experiments the insoluble material was also taken through a similar procedure prior to reconstitution.

Reconstitution of the PEG-precipitated proteins from the solubilize and from the pellet was done as follows. An asolectin suspension was prepared in the reconstitution buffer at a concentration of 40 mg/ml by first vortex-mixing the suspension for 6 min and then sonicating the material for 15 min in a bath-type sonicator. The final sample was translucent. The PEG-precipitated proteins were mixed with the desired volume of the asolectin suspension to give an asolectin/protein ratio of 10:1. The asolectin/protein mixture was subjected to freezing and thawing twice in an ethanol/solid CO_2 bath and then sonicated for 3 min. The suspension was diluted with 20 vol. of reconstitution buffer and the proteoliposomes were harvested by centrifugation of the suspension at 85 000 g for 30 min. The pellet containing the proteoliposomes was suspended in a small volume of the reconstitution buffer by passing through a 25 gauge needle. The suspension was once again sonicated for 3 min and immediately used in uptake measurements.

Uptake assay using proteoliposomes

Uptake of 5-HT or taurine in proteoliposomes was measured by a rapid filtration technique as previously described [29]. A 40 μl sample of proteoliposomes was rapidly mixed with 160 μl of the uptake medium containing radiolabelled 5-HT or taurine. After incubation for the desired time, the mixture was filtered through a Millipore filter (DAWP type; 0.65 μm pore size). The filter was then washed and the radioactivity associated with the filter was counted. The uptake medium was 10 mM-Hepes/Tris (pH 7.5), containing either 200 mM-NaCl or 200 mM-potassium gluconate. The composition of the stop buffer was 5 mM-Hepes/Tris, pH 7.5, containing 210 mM-KCl.

Correction for non-specific binding and/or transport

The uptake values were corrected for non-specific binding and/or transport, which was determined from the incorporation of radiolabelled 5-HT into proteoliposomes either in the presence of potassium gluconate or in the presence of NaCl plus excess unlabelled 5-HT. The values from these two methods were similar (2.8 ± 0.2 and 2.6 ± 0.2 pmol/15 s per mg of protein respectively at a 5-HT concentration of 100 nM). The experiments with protein-free liposomes in the presence of NaCl yielded comparable values for corresponding amount of liposomes, confirming that the experimental approach was indeed appropriate to determine the non-specific binding and/or transport. This value represented less than 15% of the total uptake measured in proteoliposomes in the presence of NaCl.

Data analysis

Uptake measurements were done in duplicate or triplicate and the variation among the replicate values was less than $\pm 5\%$ of the mean value. Each experiment was repeated two or three times and the results from these experiments are given as means $\pm$ S.E.M. Kinetic and statistical analyses were done using a commercially available computer statistics package (Statgraphics; STSC,

Fig. 1. Time course of 5-HT uptake in protein-free liposomes (a) and in proteoliposomes (b)

Brush border membranes were solubilized and the proteins in the soluble fraction were precipitated with 6% PEG. Reconstitution of the PEG-precipitated proteins into proteoliposomes was done with an asolectin/protein ratio of 10:1. Protein-free liposomes were prepared under identical conditions. Uptake of 5-HT (100 nM) was measured in the presence of either NaCl (●) or potassium gluconate (○). The values are means, with the bars representing S.E.M., for four determinations. When not shown, the error lies within the symbol.

Rockville, MD, U.S.A.). A *P* value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Solubilization and reconstitution

Rudnick & Nelson [24] and Talvenheimo & Rudnick [17] have reported, using platelet plasma membranes, that cholate at a concentration of 1% (w/v) reversibly inhibited the ability of the 5-HT transporter to bind imipramine, but was unable to solubilize the binding activity. At higher concentrations this detergent inhibited the binding activity irreversibly [24]. In the present study we investigated the ability of cholate to solubilize the 5-HT transporter from human placental brush border membranes, but as a measure of the transporter function we assayed NaCl-gradient-dependent 5-HT uptake into proteoliposomes which were reconstituted with proteins present in the soluble fraction and in the insoluble pellet. We found that, at 1% cholate, the transport activity resided predominantly in the cholate-insoluble pellet, and at 2.5% cholate the transporter was irreversibly inactivated (results not shown). These results are very similar to those reported previously by Rudnick and his co-workers [17,24], who employed imipramine binding as a measure of transporter function.

Many amino acid transport systems have been solubilized and reconstituted into proteoliposomes in a functionally active form by different methods [27,28,30–36]. In some of these methods cholate has been used as the solubilizing agent in the presence of high concentrations of urea [27,28,30–32]. Even though the exact role of urea was not investigated in these studies, it is likely that urea acts as a stabilizing agent during the detergent solubilization step. Therefore we studied the influence of urea on 5-HT transporter activity and on the ability of cholate to solubilize the transporter from the placental membranes. For this purpose, we treated the membranes with 2.5% cholate in the presence of 4 M-urea. The proteins in the soluble fraction and in the pellet were reconstituted into proteoliposomes. It was found that cholate did

not inactivate the 5-HT transporter under these conditions. More importantly, a considerable amount of the total activity (approx. 70%) was associated with the proteins in the soluble fraction, indicating that cholate in the presence of urea solubilized the transporter to a significant extent.

We also conducted preliminary experiments to determine the influence of the ionic composition of the solubilization buffer and of the protein concentration during solubilization on 5-HT transport activity in the final preparations of proteoliposomes reconstituted from the solubilized proteins. The results indicated that the transport activity was significantly higher if solubilization was done in the presence of NaCl rather than KCl or NaSCN, and that the optimal protein concentration was 2 mg/ml (results not shown). The catalytic function of the 5-HT transporter in native membrane vesicles is obligatorily dependent on Na⁺ and Cl[−], and therefore it is possible that the transport protein when bound to Na⁺ and Cl[−] assumes a conformation which is resistant to inactivation during the solubilization step. Similar experiments to determine suitable conditions for reconstitution revealed that a 6% PEG concentration was optimal to precipitate the proteins in the soluble fraction, and that the use of an asolectin/protein ratio of 10:1 in the reconstitution step resulted in maximal activity of the transporter in the proteoliposomes.

Thus the important features of the solubilization and reconstitution procedure reported in this paper are (i) solubilization of the membrane proteins by cholate in the presence of urea, (ii) removal of cholate/urea and simultaneous precipitation of proteins from the soluble fraction by PEG, and (iii) reconstitution of the PEG-precipitated proteins into proteoliposomes by a freeze-thaw/sonication procedure in the presence of K⁺. The procedure was standardized for the placental 5-HT transporter by essentially following the solubilization and reconstitution methods reported for many amino acid transport systems. The idea of including urea in the cholate solubilization step was adopted from the procedure introduced by McCormick *et al.* [27]. Removal of cholate/urea and simultaneous precipitation of proteins was originally reported by Fafournoux *et al.* [31]. The advantage of the presence of K⁺ during reconstitution

Fig. 2. Substrate specificity of the reconstituted 5-HT transporter

Solubilization of the brush border membranes and reconstitution of the solubilized proteins into proteoliposomes were done as described in the Experimental section (PEG, 6%; asolectin/protein ratio, 10:1). Uptake of radiolabelled 5-HT (100 nM) in these proteoliposomes was measured with a 15 s incubation in the presence of NaCl. The non-carrier-mediated transport component was determined as the uptake in the presence of potassium gluconate. Uptake was measured in the presence of various concentrations of 5-HT (●), dopamine (○), noradrenaline (□) and 5-hydroxytryptophan (■). The concentration range for these compounds was 10^{-8} – 10^{-2} M. Only the NaCl-dependent carrier-mediated uptake was used in calculations. Results are given as percentages of control uptake.

was realized from the work of McCormick *et al.* [30]. In addition to allowing the formation of functionally active proteoliposomes, the presence of K^+ in the reconstitution buffer was especially useful in the present study because of its role in the catalytic activity of the plasma membrane 5-HT transporter.

Characteristics of the reconstituted 5-HT transporter

Time course. Fig. 1 compares the time courses of 5-HT uptake in protein-free liposomes and in proteoliposomes. The composition of the intraliposomal medium was the same as that of the reconstitution buffer (10 mM-Hepes/Tris, 200 mM-potassium gluconate, 1 mM-magnesium gluconate, pH 7.5). Uptake of 5-HT (100 nM) was measured from a medium which contained either 200 mM-NaCl or 200 mM-potassium gluconate, buffered with 10 mM-Hepes/Tris (pH 7.5). Uptake of 5-HT into the protein-free liposomes was slow and there was no significant difference between the uptake values determined in the presence of NaCl and in the presence of potassium gluconate (Fig. 1a). In the proteoliposomes, uptake from the potassium gluconate-containing medium was again slow, comparable to the values obtained with the protein-free liposomes (Fig. 1b). However, uptake from the NaCl-containing medium was many-fold greater than from the potassium gluconate-containing medium in the proteoliposomes. With a 15 s incubation, 5-HT uptake in the presence of NaCl was 15.6 ± 0.1 pmol/mg of protein, whereas the corresponding value in the presence of potassium gluconate was only 2.6 ± 0.1 pmol/mg of protein. In addition, the time course of uptake from the NaCl medium exhibited an 'overshoot'. The intraliposomal concentration of 5-HT measured with a 5 min incubation (36.3 ± 2.3 pmol/mg of protein) was much greater than the concentration measured at equilibrium (22.5 ± 0.2 pmol/mg of protein). On the contrary, uptake from the potassium

Table 1. Influence of cations and anions on the activity of the 5-HT transporter in reconstituted proteoliposomes

Brush border membranes (20 mg of protein) were solubilized with 2.5% cholate in the presence of 4 M-urea. Solubilization was done for 30 min at 4 °C at a protein concentration of 2 mg/ml. The proteins in the soluble fraction were precipitated with 6% PEG and reconstituted into proteoliposomes with an asolectin/protein ratio of 10:1, as described in the Experimental section. The reconstitution buffer was 10 mM-Hepes/Tris, pH 7.5, containing 200 mM-potassium gluconate and 1 mM-magnesium gluconate. Uptake of 5-HT (100 nM) was measured in these proteoliposomes with a 15 s incubation. Uptake buffer was 10 mM-Hepes/Tris, pH 7.5, containing 187.5 mM of the indicated inorganic salts. The values are means $\pm$ S.E.M. for three determinations.

Salt	5-HT uptake	
	(pmol/15 s per mg of protein)	(%)
NaCl	14.9 ± 2.3	100
Sodium gluconate	2.3 ± 0.1	15
NaSCN	6.3 ± 0.1	42
NaNO ₃	3.6 ± 0.1	24
NaF	5.8 ± 0.1	39
NaI	10.5 ± 0.1	71
LiCl	2.6 ± 0.1	18
KCl	3.4 ± 0.7	23
Potassium gluconate	2.2 ± 0.1	15
RbCl	2.9 ± 0.1	20

gluconate medium slowly increased with time, but there was no evidence for active accumulation of 5-HT within the proteoliposomes. The equilibrium values measured at 180 min were similar in both the NaCl medium and the potassium gluconate medium. These data show that, unlike in the protein-free liposomes, uptake of 5-HT in the proteoliposomes showed a requirement for a NaCl gradient. These proteoliposomes were capable of accumulating 5-HT against a concentration gradient in the presence of a NaCl gradient, as shown by the overshoot.

Cation and anion specificities. In order to determine the cation and anion specificities of the reconstituted placental 5-HT transporter, we measured 5-HT uptake in the proteoliposomes from different uptake media with various cationic and anionic compositions (Table 1). First, the cation specificity was examined by comparing 5-HT uptake in the presence of chloride salts of Na^+ , Li^+ , K^+ and Rb^+ . Uptake from the medium containing LiCl, KCl or RbCl was found to be about 20% of the uptake measured in the presence of NaCl. Thus the other univalent cations failed to substitute for Na^+ . The anion specificity was then examined by comparing 5-HT uptake in the presence of sodium salts of different anions: Cl^- , SCN^- , NO_3^- , F^- , I^- and gluconate. The uptake was found to be highest in the presence of Cl^- and lowest in the presence of gluconate. The anions I^- , SCN^- and F^- could substitute for Cl^- to some extent. Thus the reconstituted 5-HT transporter shows maximal activity only when both Na^+ and Cl^- are present in the uptake medium.

Substrate and inhibitor specificities. The substrate specificity of the reconstituted placental 5-HT transporter was studied by determining the ability of unlabelled compounds to compete with radiolabelled 5-HT during the uptake process. The NaCl-dependent uptake was determined by subtracting the uptake measured in the presence of potassium gluconate from that measured in the presence of NaCl. The compounds used in this study were 5-HT, dopamine, noradrenaline and 5-hydroxytryptophan. The first three compounds are monoamines, and the fourth is the precursor for 5-HT. The concentration of radio-

Fig. 3. Inhibitor specificity of the reconstituted 5-HT transporter

The details of the experimental procedure are given in the legend to Fig. 2. Uptake of 5-HT was measured in the presence of various concentrations of imipramine (●), paroxetine (○), fluoxetine (□) and reserpine (■).

Fig. 4. Dependence of the activity of the 5-HT transporter in proteoliposomes on the concentration of Na^+

Brush border membranes were solubilized and the proteoliposomes were prepared as described in the Experimental section. The PEG concentration was 6% and the asolectin/protein ratio was 10:1. Uptake of 5-HT (150 nM) was measured with a 15 s incubation in the presence of various concentrations of Na^+ (0–150 mM) and a fixed concentration of Cl^- (150 mM). Uptake buffers were prepared by appropriately mixing two solutions containing either 200 mM-NaCl or 200 mM-LiCl. Both solutions were buffered with 10 mM-Hepes/Tris, pH 7.5. The values are means, with bars representing S.E.M., for four determinations.

labelled 5-HT was 100 nM and that of the unlabelled compounds was varied between 10^{-8} and 10^{-2} M. The data in Fig. 2 show that the uptake system was highly specific for 5-HT. Dopamine and noradrenaline also interacted with the system, but the affinity of the system for these compounds was 2–3 orders of magnitude lower than that for 5-HT. The IC_{50} values for dopamine and noradrenaline were 100 μM and 760 μM respectively. 5-Hydroxytryptophan showed the least ability to interact with the uptake system (IC_{50} 4 mM).

Fig. 5. Dependence of the activity of the 5-HT transporter in proteoliposomes on the concentration of Cl^-

Brush border membranes were solubilized and the proteoliposomes were prepared as described in the Experimental section. The PEG concentration was 6% and the asolectin/protein ratio was 10:1. Uptake of 5-HT (150 nM) was measured with a 15 s incubation in the presence of various concentrations of Cl^- (0–150 mM) and a fixed concentration of Na^+ (150 mM). Uptake buffers were prepared by appropriately mixing two solutions containing either 200 mM-NaCl or 200 mM-sodium gluconate. Both solutions were buffered with 10 mM-Hepes/Tris, pH 7.5. The values are means for three determinations.

The inhibitor specificity was then studied using a similar approach, in which the potency of various drugs to decrease NaCl-dependent 5-HT uptake was determined. In these experiments the concentration of the drugs was varied between 10^{-9} and 10^{-4} M. The data given in Fig. 3 show the imipramine, paroxetine and fluoxetine were potent inhibitors of the reconstituted uptake system, with IC_{50} values of 0.1 μM , 0.5 μM and 1 μM respectively. Reserpine failed to inhibit the system even at a concentration as high as 3.16 μM .

Na^+ and Cl^- kinetics. Since the results given in Fig. 1 and Table 1 showed that the reconstituted 5-HT transport system has an absolute requirement for Na^+ and Cl^- for its catalytic activity, we studied the kinetics of this Na^+ and Cl^- requirement. First, we measured the 5-HT uptake rates (150 nM-5-HT, 15 s incubation) at various concentrations (0–150 mM) of external Na^+ , while keeping the external Cl^- concentration constant at 150 mM (Fig. 4). The dependence of the uptake rate on the concentration of Na^+ was hyperbolic, indicating that the coupling ratio of Na^+ /5-HT is 1:1. This is supported by the observation that the plot of v versus $v/[\text{Na}^+]$ was linear (Fig. 4, inset). We also investigated the Cl^- kinetics of the reconstituted 5-HT transporter by measuring the rates of NaCl-dependent 5-HT uptake at various concentrations of external Cl^- (0–150 mM) while keeping the concentration of external Na^+ constant at 150 mM. The relationship between the uptake rate and the Cl^- concentration was hyperbolic (Fig. 5), and the plot of v versus $v/[\text{Cl}^-]$ was linear (Fig. 5, inset), indicating that one Cl^- was involved in the transport of one 5-HT molecule.

Electrogenicity. 5-HT exists as a cation at pH 7.5. The experiments described above show that one Na^+ ion and one Cl^- ion are involved in the transport of one 5-HT molecule in the reconstituted proteoliposomes. In addition, K^+ present in the intraliposomal medium is also expected to participate as a transportable ion in the catalytic process. Therefore, it was of

interest to investigate whether the uptake of 5-HT into the proteoliposomes via the reconstituted placental 5-HT transporter was electrogenic or electroneutral. This was done by studying the influence of a valinomycin-induced inside-negative K^+ -diffusion potential on the uptake. 5-HT (100 nM) uptake was measured in the presence and absence of 10 μ M-valinomycin. Since the proteoliposomes were reconstituted in a medium containing 200 mM- K^+ and the uptake buffer contained 200 mM-NaCl but no K^+ , there was an outwardly directed K^+ gradient when the uptake of 5-HT was measured ($[K^+]_i = 200$ mM; $[K^+]_o = 40$ mM). Under these conditions, valinomycin was expected to generate an inside-negative K^+ -diffusion potential. However, it was found that such a manoeuvre failed to influence 5-HT uptake (control, 7.02 ± 0.34 pmol/10 s per mg of protein; membrane potential, 7.5 ± 0.25 pmol/10 s per mg of protein; $P > 0.05$), indicating that the uptake process was electrically silent. That an inside-negative membrane potential was indeed generated under these conditions was evident because the uptake of taurine (0.3 μ M) was significantly stimulated by valinomycin under identical conditions (control, 1.41 ± 0.02 pmol/10 s per mg of protein; membrane potential, 2.08 ± 0.02 pmol/10 s per mg of protein; $P < 0.001$). The reconstituted proteoliposomes also possess the placental taurine transporter in a catalytically active form, and the uptake of taurine in these proteoliposomes occurs with a Na^+/Cl^- /taurine stoichiometry of 2:1:1 (S. Ramamoorthy, P. Kulanthavel, F. H. Leibach, V. B. Mahesh & V. Ganapathy, unpublished work). This stoichiometry renders the taurine uptake process electrogenic, and an inside-negative membrane potential is expected to enhance the uptake rate.

The Na^+/Cl^- /5-HT stoichiometry for the reconstituted placental 5-HT transporter is 1:1:1. The negligible effect of the valinomycin-induced inside-negative K^+ -diffusion potential on 5-HT uptake in the proteoliposomes indicates that the reconstituted transporter operates in an electrically silent manner. This suggests that each transport cycle involves countertransport of one positive charge. The countertransported cationic species is most likely K^+ , because the presence of internal K^+ stimulates 5-HT uptake in placental brush border membrane vesicles [11,14]. The role of K^+ as the countertransport ion has also been demonstrated for the platelet [6] and brain [8] 5-HT transporters.

5-HT kinetics. The dependence of the uptake rate on the concentration of 5-HT was investigated in the reconstituted proteoliposomes. In this experiment, uptake was measured in a 15 s incubation over a 5-HT concentration range of 0.05–2 μ M. When the uptake rate was plotted against the 5-HT concentration, the plot was hyperbolic (Fig. 6), indicating that a single transport system participates in the uptake of 5-HT. This conclusion was supported by the linear Eadie-Hofstee plot [uptake rate versus (uptake rate/5-HT concentration)] (Fig. 6, inset). The kinetic parameters (K_t , apparent Michaelis-Menten constant and V_{max} , the maximal velocity) for the transport system were calculated from the plot. The K_t was 0.34 ± 0.01 μ M and the V_{max} was 51.6 ± 1.0 pmol/15 s per mg of protein. The affinity of the native transporter for 5-HT varies depending upon the ionic composition of the intra- and extra-vesicular media [14]. We have previously reported that in native brush border membrane vesicles the K_t for 5-HT is 0.24 μ M when the $[NaCl]_o$ is 120 mM, the $[K^+]_i$ is 50 mM and $pH_i = pH_o = 7.5$ [14]. The kinetic parameters for the reconstituted transporter were calculated in the present study with $[NaCl]_o$ of 160 mM, $[K^+]_i$ of 200 mM and $pH_i = pH_o = 7.5$. Thus the affinity of the reconstituted transporter for 5-HT is comparable to that of the native transporter.

In order to determine if the solubilization and reconstitution procedure selectively enriches the 5-HT transporter in the proteoliposomes, we compared the SDS/PAGE patterns of

Fig. 6. Dependence of the uptake rate on the concentration of 5-HT in proteoliposomes

Brush border membranes were solubilized and the proteoliposomes were prepared as described in the Experimental section. The PEG concentration was 6% and the asolectin/protein ratio was 10:1. Uptake of 5-HT was measured with a 15 s incubation over the 5-HT concentration range of 0.05–2 μ M. The values are means, with the bars representing S.E.M., for four determinations. When not shown, the error lies within the symbol. Inset: Eadie-Hofstee plot.

proteins in the native brush border membranes and in the proteoliposomes. Even though there were significant differences in the relative intensities of various protein bands between the two samples, the proteoliposomes still contained most of the proteins originally present in the brush border membranes (results not shown). These results demonstrate that there was no selective removal of proteins unrelated to the 5-HT transporter during the solubilization and reconstitution procedure.

In conclusion, the present study represents the first demonstration of the successful solubilization and reconstitution of the plasma membrane 5-HT transporter in a functionally active form. The characteristics of the reconstituted transporter are similar to those of the native transporter. The simple and reliable solubilization and reconstitution procedure reported here will be very useful in future studies involving further characterization of the 5-HT transporter, not only in the placenta but also in the platelet and the brain.

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