Expression of a Cocaine-Sensitive Norepinephrine Transporter in the Human Placental Syncytiotrophoblast†

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ABSTRACT: Maternal-facing brush border membrane vesicles isolated from normal term human placentas were found to accumulate norepinephrine in a concentrative manner in the presence of an inwardly directed NaCl gradient. Both Na^+ and Cl^- were obligatory for maximal uptake. The NaCl-dependent norepinephrine uptake was further stimulated by the presence of K^+ or an acidic pH in the intravesicular medium. The uptake process was electrogenic, being stimulated by an inside-negative membrane potential, and this characteristic was observed in the absence as well as in the presence of K^+ inside the vesicles. Kinetic analyses revealed that one Na^+ and one Cl^- were involved per transport of one norepinephrine molecule. The apparent Michaelis–Menten constant for norepinephrine was 104 ± 5 nM. The uptake process exhibited higher affinity for dopamine than for norepinephrine but had low affinity for serotonin and histamine. The uptake of norepinephrine was inhibited very effectively by nomifensine, desipramine, imipramine, and cocaine, but much less effectively by bupropion and GBR 12909. Northern blot analysis with the cDNA of the human (SK-N-SH cell) norepinephrine transporter as the probe revealed that the human placenta contained two mRNAs, 5.8 and 3.6 kb in size, which hybridized to the probe. The JAR human placental choriocarcinoma cells were found unable to accumulate norepinephrine in a NaCl-dependent manner. These cells were also found not to contain mRNAs which hybridized to the norepinephrine cDNA probe in northern blot. It is concluded that the human placental syncytiotrophoblast expresses a cocaine-sensitive norepinephrine transporter and that these findings may be directly relevant and important to the clinical complications of maternal cocaine abuse during pregnancy.

There are three distinct monoamine transporters, the serotonin transporter, the dopamine transporter, and the norepinephrine transporter, that are localized in the plasma membrane of only a few cell types. The nature and function of these transporters have received considerable attention in the neuronal tissue where the transporters serve to terminate monoaminergic signal transmission by catalyzing rapid reuptake of the monoamines from the synaptic cleft (Iversen, 1975). All three transporters have been cloned recently (Blakeley et al., 1991; Giros et al., 1991; Hoffman et al., 1991; Kilty et al., 1991; Pacholczyk et al., 1991; Shimada et al., 1991; Usdin et al., 1991). The catalytic mechanism of all three transporters share many common features which include energization by transmembrane Na^+ and Cl^- gradients, activation by intracellular K^+, and recognition of the cationic form of the respective monoamine as the substrate. However, the three transporters can be distinguished from one another by their monoamine substrate specificities and by their differential sensitivities to a wide spectrum of transport antagonists.

Interestingly, only very few nonneuronal tissues exhibit monoamine transporter activities with characteristics similar to those of the neuronal monoamine transporters. We have recently demonstrated that brush border membranes isolated from normal term human placentas possess an active transport mechanism specific for serotonin (Balkovetz et al., 1989). These membranes, derived from the syncytiotrophoblast cells of the placenta, are in direct contact with maternal blood and constitute the first barrier between maternal and fetal circulations. The catalytic and pharmacologic properties of the serotonin transporter in these membranes are remarkably similar to those of the serotonin transporter in the neuronal tissue (Balkovetz et al., 1989; Cool et al., 1990a,b,c, 1991; Ganapathy et al., 1989). In addition, these brush border membrane vesicles also have the ability to accumulate dopamine against a concentration gradient in the presence of a transmembrane NaCl gradient (Ramamoorthy et al., 1992b). This transport system could be distinguished from the previously described serotonin transporter on the basis of substrate and inhibitor specificities. Surprisingly however, even though the transport system showed the highest affinity for dopamine among the three monoamines, its pharmacologic properties had no resemblance to those of the dopamine transporter described in the neuronal tissue. The sensitivity of dopamine uptake to various inhibitors indicated that the transport system responsible for this uptake may be the norepinephrine transporter rather than the dopamine transporter.

The present study was undertaken to determine whether the placental brush border membrane vesicles possess the ability to transport norepinephrine, and the results of the study show that these membrane vesicles are indeed able to accumulate norepinephrine in a NaCl-dependent manner. We report here the characteristics of this norepinephrine uptake and the inhibitor specificity of the uptake process, including its sensitivity to cocaine and various antidepressants. We also provide evidence in this paper for the presence of mRNA in the human placenta which hybridizes to the cDNA encoding the human (SK-N-SH cell) norepinephrine transporter under

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high stringency conditions. These results support the conclusion that the human placental syncytiotrophoblast expresses a cocaine- and antidepressant-sensitive norepinephrine transporter and that this transporter is responsible for the uptake of norepinephrine as well as for the uptake of dopamine in placental brush border membrane vesicles.

MATERIALS AND METHODS

Materials. *levo*-[7-^{3}H]Norepinephrine (specific radioactivity, 11.4 Ci/mmol), 5-[1,2-^{3}H(N)]hydroxytryptamine (serotonin) binoxalate (specific radioactivity, 23.4 Ci/mmol), [2-^{3}H(N)]taurine (specific radioactivity, 25.6 Ci/mmol) and [*phenyl*-^{14}C]tetraphenylphosphonium (TPP) bromide (specific radioactivity, 19.2 mCi/mmol) were purchased from Du Pont–New England Nuclear (Boston, MA). Unlabeled monoamines, imipramine, desipramine, cocaine, and iproniazid were obtained from Sigma (St. Louis, MO). FCCP[1] was from Aldrich (Milwaukee, WI). Nomifensine, bupropion, and GBR 12909 were purchased from Research Biochemicals (Natick, MA). The human placental choriocarcinoma cell line (JAR) was obtained from the American Type Culture Collection. RPMI 1640 medium, fetal bovine serum, penicillin–streptomycin, and fungizone were from GIBCO Labs (Grand Island, NY).

Preparation of Human Placental Brush Border Membrane Vesicles. A Mg^{2+}-aggregation method was used to prepare the maternal-facing brush border membrane vesicles from normal term human placentas (Balkovetz et al., 1986; Kulanthaivel et al., 1990). The membrane vesicles were normally preloaded with a 20 mM Mes/Tris buffer (pH 6.5) containing 280 mM mannitol or 80 mM mannitol plus 100 mM potassium gluconate. In some experiments, the pH of the preloading buffer was 7.5 in which case the buffer was 20 mM Hepes/Tris. In kinetic experiments dealing with the stoichiometry of Na^+ and Cl^-, the composition of the preloading buffer was 20 mM Mes/Tris (pH 6.5), 100 mM potassium gluconate plus 400 mM mannitol. Protein concentration in final preparations was adjusted to 6 mg/mL. The membrane vesicles were then stored in liquid N_2 in small aliquots until used.

Uptake Measurement in Membrane Vesicles. Uptake of radiolabeled norepinephrine or taurine into placental brush border membrane vesicles was determined by a rapid filtration method as described previously (Ganapathy et al., 1981). Uptake was initiated by mixing 40 μL of membrane suspension (240 μg of membrane protein) with 160 μL of uptake buffer containing radiolabeled substrate. After incubation for a desired time at room temperature (22 °C), uptake was terminated by the addition of 3 mL of ice-cold stop buffer (5 mM Mes/Tris, 160 mM KCl, pH 6.5) followed by vacuum filtration on a Millipore filter (DAWP type, 0.65-μm pore size). The membrane vesicles retained on the filter were further washed three times with 3 mL of the stop buffer. The radioactivity associated with the membrane vesicles was then determined by liquid scintillation spectrometry. The uptake medium, in most cases, was 20 mM Mes/Tris buffer (pH 6.5), containing 140 mM NaCl. The composition of the uptake medium was modified in some experiments as required.

Uptake of radiolabeled TPP was determined by a similar procedure except that GF/F glass fiber filter which had been presoaked for 45 min in 0.3% polyethyleneimine was used in place of Millipore filter during filtration.

Cell Culture and Uptake Measurement in Cells. The JAR placental choriocarcinoma cells were cultured as confluent monolayers in 35-mm plastic dishes as described previously (Cool et al., 1991; Kulanthiaivel et al., 1991). Uptake of serotonin and norepinephrine in these cells was determined using radiolabeled substrates. Prior to initiation of uptake measurements, the cells were incubated with 100 μM iproniazid (an inhibitor of monoamine oxidase) for 30 min to prevent cellular metabolism of transported monoamines (Cool et al., 1991). The uptake medium was 25 mM Hepes/Tris (pH 7.5) containing 140 mM NaCl (or 140 mM choline chloride), 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. After incubation of the cells with the radiolabeled substrate for a desired time, the medium was aspirated and the cells were washed three times with the uptake medium. The radioactivity associated with the cells was determined as described previously (Cool et al., 1991).

Placental and JAR Cell RNA Hybridization. Agarose gel-purified cDNA (50 ng) encoding the human (SK-N-SH cell) norepinephrine transporter was radiolabeled by random priming as previously described (Pacholczyk et al., 1991) and purified from free radionucleotides by size-exclusion chromatography (Nuc-Trap, Stratagene). Poly(A)-enriched RNA from human placenta (1 μg, Clontech), JAR cells (1 μg), and SK-N-SH total RNA (5 μg) were size-fractionated on a denaturing formaldehyde agarose gel and transferred to activated nylon membrane (Zetaprobe, Bio-Rad) by vacuum blotting. Following overnight prehybridization of the membrane at 42 °C in 50% formamide, 5% dextran sulfate, 5× SSPE, 1% SDS, and 0.5 mg/mL heparin in 20 mL, hybridizations were initiated by the addition of the cDNA probe and continued at 42 °C for 24 h. The blot was washed twice at 22 °C in 2× SSPE, 0.1% SDS, followed by a 1-h wash at 65 °C in 0.1× SSPE, 0.1% SDS, and exposed to autoradiographic film with an intensifying screen for 5 days. Sizes of hybridizing RNAs are based on parallel electrophoresis of RNA standards (Gibco-BRL).

Data Analysis. Uptake measurements in membrane vesicles were performed in duplicate or triplicate, using two to three different membrane preparations. The results are given as means ± SE for these replicate determinations. Kinetic analyses were conducted using a commercially available computer program (Statgraphics STSC, Rockville, MD).

RESULTS

Evidence for Concentrative Uptake of Norepinephrine in Human Placental Brush Border Membrane Vesicles. Figure 1 describes the time course of norepinephrine uptake in placental brush border membrane vesicles in the presence of an inwardly directed NaCl gradient or KCl gradient (initial zero-trans). Uptake of norepinephrine was very slow in the presence of a KCl gradient, and there was no evidence for active accumulation of the monoamine inside the vesicles. On the contrary, the uptake was very rapid when the KCl gradient was replaced with a NaCl gradient. The initial uptake rates were many times higher in the presence of a NaCl gradient than in the presence of a KCl gradient. Moreover, there was a transient accumulation of the monoamine inside the vesicles against a concentration gradient as evidenced from the overshoot.

Influence of Monovalent Cations and Anions on Norepinephrine Uptake. In order to determine the specificity of Na^+ for the activation of norepinephrine uptake, the initial

[1] Abbreviations: Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyetheyl)-1-piperazineethanesulfonic acid; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; NMG, *N*-methylglucamine; TPP, tetraphenylphosphonium.

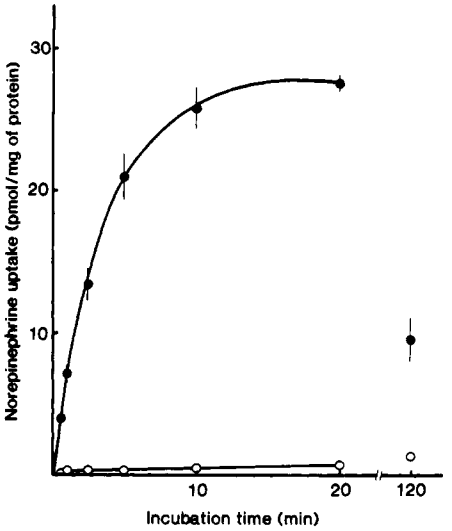

FIGURE 1: Effects of an inwardly directed NaCl gradient or KCl gradient on norepinephrine uptake. Human placental brush border membrane vesicles were preloaded with 20 mM Mes/Tris buffer, pH 6.5, containing 280 mM mannitol. Uptake buffer was 20 mM Mes/Tris, pH 6.5, containing either 140 mM NaCl (●) or 140 mM KCl (○). The concentration of norepinephrine was 0.2 μM. Values are means ± SE for four determinations.

Table I: Influence of Monovalent Cations and Anions on the Uptake of Norepinephrine[a]

inorganic salt	norepinephrine uptake (pmol/mg of protein per 15 s)	(%)
	Influence of Cations	
NaCl	1.63 ± 0.21	100
KCl	0.24 ± 0.01	15
LiCl	0.24 ± 0.02	15
CsCl	0.35 ± 0.02	22
NMGCl	0.31 ± 0.01	19
	Influence of Anions	
NaCl	1.63 ± 0.21	100
NaF	0.17 ± 0.01	10
sodium gluconate	0.11 ± 0.01	7
Na NO_3	0.53 ± 0.09	33
NaSCN	1.32 ± 0.16	81

[a] Human placental brush border membrane vesicles were preloaded with 20 mM Mes/Tris buffer, pH 6.5, containing 280 mM mannitol. Uptake buffer was 20 mM Mes/Tris, pH 6.5, containing the indicated inorganic salt. Final concentration of the inorganic salts during uptake measurement was 100 mM. Uptake of norepinephrine (0.2 μM) was measured with a 15-s incubation. The data represent means ± SE for four determinations.

uptake rate of the monoamine was determined in the presence of an inwardly directed gradient of a number of monovalent cations. These cations were present in the form of chloride salts and therefore there was also an inwardly directed Cl^- gradient in each case. The data given in Table I show that the uptake was maximal in the presence of NaCl. When NaCl was substituted with the chloride salts of other monovalent cations such as K^+, Li^+, Cs^+, and NMG^+, the uptake rate was decreased markedly to about one-fifth of the value obtained in the presence of NaCl. Similar experiments were done to determine the anion specificity for the activation process by measuring the initial uptake of norepinephrine in the presence of sodium salts of various monovalent anions in the place of NaCl in the uptake medium (Table I). Substitution of Cl^- with F^- or gluconate almost totally abolished the uptake, showing that the presence of Na^+ alone is not enough to activate norepinephrine uptake. The presence of Na^+ as well as Cl^- is mandatory for maximal uptake. The anions SCN^- and NO_3^- could partially substitute for Cl^- in transport assays.

Table II: Influence of pH, Transmembrane pH Gradient, and Intravesicular K^+ on Norepinephrine Uptake[a]

intravesicular pH	extravesicular pH	intravesicular K^+	norepinephrine uptake (pmol/mg per 15 s)
7.5	7.5	absent	0.18 ± 0.01
7.5	7.5	present	2.58 ± 0.03
6.5	6.5	absent	1.02 ± 0.08
6.5	6.5	present	3.01 ± 0.02
6.5	7.5	absent	0.68 ± 0.03
6.5	7.5	present	2.51 ± 0.03

[a] Human placental brush border membrane vesicles were preloaded with four different buffers: 20 mM Mes/Tris (pH 6.5) containing 280 mM mannitol, 20 mM Hepes/Tris (pH 7.5) containing 280 mM mannitol, 20 mM Mes/Tris (pH 6.5) containing 100 mM potassium gluconate plus 80 mM mannitol, and 20 mM Hepes/Tris (pH 7.5) containing 100 mM potassium gluconate plus 80 mM mannitol. Uptake of norepinephrine (0.2 μM) was measured with a 15-s incubation from an uptake medium containing NaCl to give a final concentration of 110 mM during uptake measurement. Uptake medium was buffered with either 20 mM Mes/Tris (pH 6.5) or 20 mM Hepes/Tris (pH 7.5 and 7.7). Extravesicular pH values represent the final pH of the uptake medium after mixing the membrane vesicles with the uptake buffer. The data represent means ± SE for three determinations.

Influence of pH, Transmembrane H^+ Gradient, and Intravesicular K^+ on Norepinephrine Uptake. The effect of pH on the NaCl gradient-dependent norepinephrine uptake was first studied by changing the pH of both the extravesicular and the intravesicular media. There was no transmembrane pH gradient under these conditions. The uptake rate was found to be stimulated many-fold by changing the pH from 7.5 to 6.5 (0.18 ± 0.01 vs 1.02 ± 0.08 pmol/mg of protein per 15 s) (Table II). To determine whether this simulatory effect of acid pH was due to acidification of the intravesicular medium or that of the extravesicular medium, the uptake rate was measured at a fixed extravesicular pH (7.5), but at different intravesicular pHs. The uptake rate was 3 to 4 times higher at an intravesicular of pH 6.5 than at pH 7.5. Thus, irrespective of whether or not a transmembrane H^+ gradient is present, an intravesicular acidic pH (i.e., intravesicular H^+) activates NaCl gradient-dependent norepinephrine uptake. In these experiments, the intravesicular pH was altered by preloading the membrane vesicles with buffers of desired pH. The preloading procedure involved incubation of the membrane vesicles with desired buffer at room temperature for 1 h, followed by centrifugation and suspension of the resulting membrane pellet in a small volume of the same buffer. It was assumed that the pH of the intravesicular medium became equal to the pH of the incubation buffer by this procedure.

The influence of intravesicular K^+ was then investigated (Table II). The NaCl gradient-dependent uptake of norepinephrine was stimulated markedly by the presence of K^+ inside the vesicles. This stimulation was evident at pH 7.5 as well as at pH 6.5 and also in the presence of a transmembrane pH gradient (intravesicular pH < extravesicular pH). However, the extent of stimulation was influenced by intravesicular pH. The presence of K^+ inside the vesicles stimulated the uptake rate 14-fold when the intravesicular pH was 7.5. But, the stimulation was only 3–4-fold when the intravesicular pH was 6.5. These results suggest that a common mechanism may be involved in the activation of norepinephrine uptake by intravesicular K^+ and H^+ and that these ions may compete for a common binding site on the transporter that is responsible for activation.

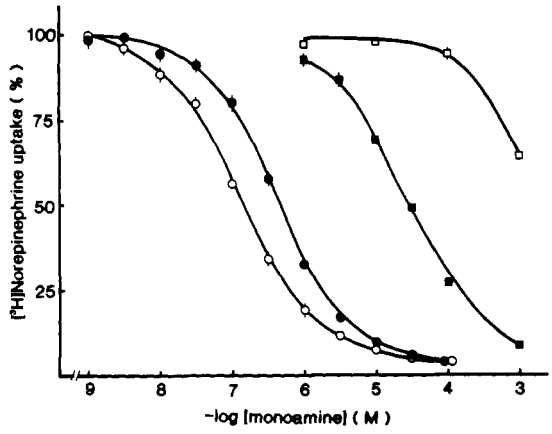

FIGURE 2: Substrate specificity of the placental norepinephrine transport system. Human placental brush border membrane vesicles were preloaded with 20 mM Mes/Tris buffer, pH 6.5, containing 280 mM mannitol. Uptake of [^{3}H]norepinephrine (0.2 μM) was measured with a 15-s incubation from an uptake medium (pH 6.5) containing NaCl at a final concentration of 112 mM. Unlabeled substrates were present in the uptake medium at varying concentrations (range, 10^{-9}–10^{-3} M). The control uptake (100%) measured in the absence of unlabeled substrates was 1.47 ± 0.06 pmol/mg of protein per 15 s. Unlabeled substrates: dopamine (○); norepinephrine (●); serotonin (■); histamine (□). Values are means ± SE for four determinations.

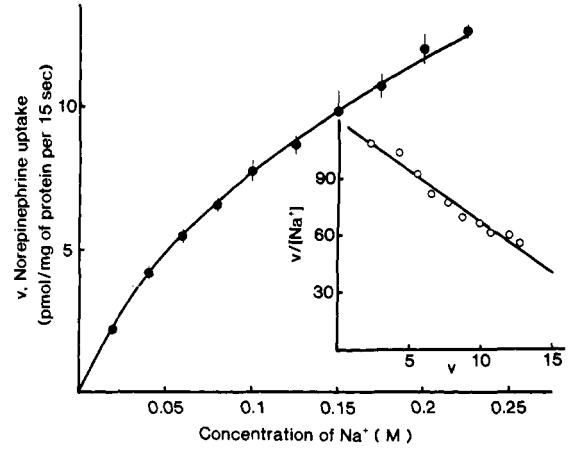

FIGURE 3: Na^+ dependence of the placental norepinephrine transport system. Human placental brush border membrane vesicles were preloaded with 20 mM Mes/Tris buffer (pH 6.5) containing 100 mM potassium gluconate and 400 mM mannitol. Uptake of norepinephrine (0.15 μM) was measured with a 15-s incubation from an uptake medium containing varying concentrations of Na^+ (0–225 mM) and a fixed concentration of Cl^- (225 mM). Uptake buffers were prepared by appropriately mixing two solutions containing 300 mM NaCl or 300 mM LiCl, buffered with 20 mM Mes/Tris, pH 6.5. Only the Na^+-dependent uptake rates (uptake rate in the presence of Na^+ minus uptake rate in the absence of Na^+) were used in the data analysis. Values are means ± SE for four determinations.

Substrate Specificity of the Norepinephrine Transport System. To study the substrate specificity of the transport system that is responsible for the uptake of norepinephrine, competition experiments were performed to see if other monoamines inhibit [^{3}H]norepinephrine uptake by competing for the uptake process (Figure 2). The monoamines (unlabeled) tested were norepinephrine, dopamine, serotonin, and histamine. As expected, unlabeled norepinephrine competed with labeled norepinephrine for the uptake mechanism and the IC_{50} (50% inhibitory concentration) for the process was 0.48 μM. Interestingly, dopamine was more potent than norepinephrine in inhibiting the uptake of [^{3}H]norepinephrine. The IC_{50} for dopamine was 0.15 μM, about 3 times lower than that for norepinephrine. Serotonin with an IC_{50} of 30 μM and histamine with an IC_{50} of greater than 1 mM were very weak in competing for the norepinephrine uptake process. The concentration of radiolabeled norepinephrine in these experiments was 200 nM. The IC_{50} values from these experiments were used to determine the apparent inhibition constants (K_i) for dopamine, norepinephrine, and serotonin according to the method described by Cheng and Prusoff (1973), employing a value of 104 nM for the Michaelis–Menten constant (K_t) (see below). The K_i values were found to be 50 nM, 160 nM, and 10 μM, respectively.

Na^+ and Cl^- Kinetics of the Norepinephrine Transport System. The dependence of the norepinephrine uptake rate on the concentration of Na^+ in the uptake of medium was determined in the presence of a fixed concentration of Cl^-. The relationship was hyperbolic and the Eadie–Hofstee-type plot, i.e., uptake rate vs uptake rate/Na^+ concentration, was linear (Figure 3), suggesting involvement of one Na^+ per transport of one norepinephrine molecule. Similar experiments on the dependence of the norepinephrine uptake rate on the concentration of Cl^- done in the presence of a fixed concentration of Na^+ revealed that one Cl^- was involved per transport of one norepinephrine molecule (data not shown). These results suggest a Na^+:Cl^-:norepinephrine stoichiometry of 1:1:1. The $K_{0.5}$ value (the concentration of the ion necessary to elicit 50% of the maximal effect) is approximately 200 mM for Na^+ and 160 mM for Cl^-.

Influence of an Inside-Negative Membrane Potential on Norepinephrine Uptake. To determine whether the NaCl-dependent norepinephrine uptake in placental brush border membrane vesicles is electrogenic or electroneutral, the influence of an inside-negative membrane potential on the initial uptake rate of norepinephrine was studied (Table III). The inside-negative membrane potential was generated by FCCP, a proton ionophore, in the presence of an outwardly directed H^+ gradient across the vesicular membrane. The FCCP-induced H^+ diffusion potential stimulated the NaCl-dependent norepinephrine uptake, suggesting that the uptake process is electrogenic and that the process results in the net transfer of positive charge(s) into the vesicles. The electrogenic nature of the uptake of norepinephrine was seen in the absence as well as in the presence of intravesicular K^+. To provide evidence for the generation of the inside-negative membrane potential under these experimental conditions, the NaCl-dependent uptake of taurine and the uptake of the lipophilic cation TPP were determined in parallel. The taurine transporter in human placental brush border membrane vesicles is electrogenic, and the uptake process results in the transfer of net positive charge(s) into the vesicles (Karl & Fisher, 1990; Miyamoto et al., 1988) and is therefore expected to be stimulated by the FCCP-induced, inside-negative H^+-diffusion potential. Similarly, uptake of TPP whose accumulation inside the vesicles is influenced solely by the potential difference across the membrane is also expected to be enhanced under these conditions. The data given in Table III show that the experimental conditions which stimulated norepinephrine uptake also stimulated the uptake of taurine and the uptake of TPP, thus supporting the validity of the conclusion that the norepinephrine transport system in human placental brush border membrane vesicles is electrogenic.

Influence of Norepinephrine Concentration on the Uptake Rate. The relationship between the initial uptake rate and the norepinephrine concentration over a range of 10–250 nM was hyperbolic (Figure 4). The experimental data obeyed Michaelis–Menten kinetics, as evidenced by the linear Eadie–Hofstee plot (uptake rate/norepinephrine concentration vs uptake rate). These results suggest participation of a single,

Table III: Influence of an Inside-Negative H^+-Diffusion Potential on the Uptake of Norepinephrine, Taurine, and TPP[a]

	norepinephrine uptake		taurine uptake		TPP uptake	
experimental condition	(pmol/mg per 15 s)	(%)	(pmol/mg per 15 s)	(%)	(pmol/mg per 30 s)	(%)
absence of intravesicular K^+						
control	0.46 ± 0.01	100	0.58 ± 0.01	100	166 ± 2	100
FCCP	0.65 ± 0.01	141	1.00 ± 0.02	172	343 ± 4	207
presence of intravesicular K^+						
Control	1.01 ± 0.06	100	0.99 ± 0.01	100	155 ± 2	100
FCCP	1.75 ± 0.03	173	1.62 ± 0.01	164	259 ± 3	167

[a] Human placental brush border membrane vesicles were preloaded with 20 mM Mes/Tris (pH 6.5) containing either 280 mM mannitol (absence of intravesicular K^+) or 100 mM potassium gluconate plus 80 mM mannitol (presence of intravesicular K^+). Uptake of norepinephrine (0.15 μM), taurine (0.2 μM), or TPP (6 μM) was measured with either a 15-s incubation (norepinephrine and taurine) or a 30-s incubation (TPP) from an uptake medium which was 20 mM Hepes/Tris (pH 7.9) containing 140 mM NaCl. After the membrane vesicles were mixed with the uptake medium, final extravesicular pH was 7.5 and final concentration of NaCl was 110 mM. An inside-negative H^+-diffusion potential was generated by the addition of FCCP at a final concentration of 20 μM. The data represent means ± SE for three determinations.

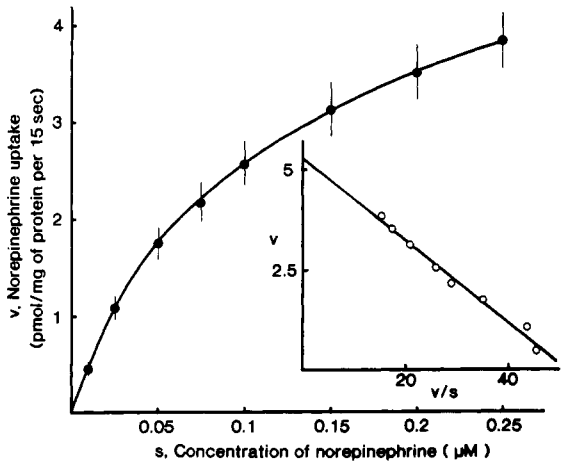

FIGURE 4: Kinetic analysis of the placental norepinephrine transport system. Human placental brush border membrane vesicles were preloaded with 20 mM Mes/Tris buffer (pH 6.5) containing 100 mM potassium gluconate and 80 mM mannitol. Uptake medium was 20 mM Mes/Tris buffer (pH 6.5) containing 140 mM NaCl. Final concentration of NaCl during uptake measurement was 112 mM. Uptake of [^{3}H]norepinephrine (concentration range, 10–250 nM) was measured with a 15-s incubation. Nonmediated uptake was calculated at each concentration of [^{3}H]norepinephrine from the uptake of radiolabel measured in the presence of excess amount (0.5 mM) of unlabeled norepinephrine. These values were subtracted from respective total uptake values to determine mediated uptake at each norepinephrine concentration. Values are means ± SE for six determinations.

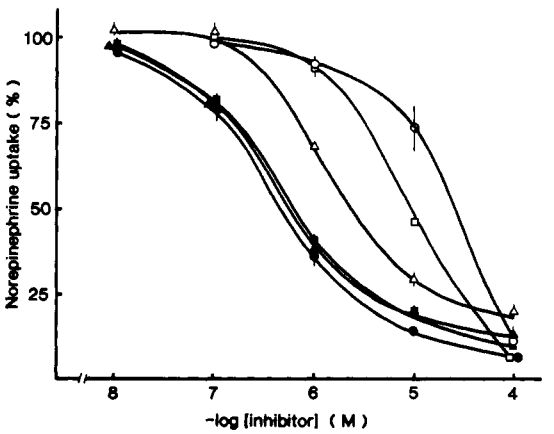

FIGURE 5: Inhibitor specificity of the placental norepinephrine transport system. Human placental brush border membrane vesicles were preloaded with 20 mM Mes/Tris buffer (pH 6.5) containing 280 mM mannitol. Uptake of norepinephrine (0.2 μM) was measured with a 15-s incubation from an uptake medium (pH 6.5) containing NaCl at a final concentration of 112 mM. Inhibitors were present in the uptake medium at varying concentrations (range 10^{-8}–10^{-4} M). The control uptake (100%) measured in the absence of inhibitors was 1.22 ● 0.07 pmol/mg of protein per 15s. Inhibitors: nomifensine (●); desipramine (■); imipramine (▲); GBR 12909 (○); bupropion (□); cocaine (△). Values are means ± SE for four determinations.

saturable transport system in the uptake of norepinephrine in human placental brush border membrane vesicles. The Michaelis–Menten constant (K_t) for norepinephrine was 104 ± 5 nM and the maximal velocity (V_{max}) for the system was 5.3 ± 0.2 pmol/mg of protein per 15 s.

Inhibitor Specificity of the Norepinephrine Transport System. The effects of various monoamine uptake inhibitors on the uptake of norepinephrine were then studied (Figure 5). Among the compounds employed, desipramine, imipramine, and nomifensine were the most potent inhibitors of norepinephrine uptake in placental brush border membrane vesicles and the IC_{50} for these inhibitors was in the range of 0.45–0.60 μM. Cocaine was also an effective inhibitor of norepinephrine uptake with an IC_{50} of 2.3 μM. Bupropion and GBR 12909 were weak inhibitors with IC_{50} values of about 10 and 30 μM, respectively.

Uptake of Norepinephrine in JAR Placental Choriocarcinoma Cells. The JAR cells, grown as confluent monolayers on impermeable plastic support, express a high affinity, NaCl-dependent serotonin transporter whose characteristics are similar to those of the serotonin transporter present in the normal placental brush border membrane (Cool et al., 1991). These cells, however, do not have the ability to transport dopamine in a NaCl-dependent manner (Ramamoorthy et al., 1992b). In the present study, the ability of the JAR cells to transport norepinephrine was investigated and it was found that the uptake of norepinephrine, whether measured in the absence of NaCl or in its presence, remained the same in these cells (data not shown). The JAR placental choriocarcinoma cells thus do not express the NaCl-dependent norepinephrine transport mechanism that is observed in normal placental brush border membrane vesicles.

Northern Blot. Expression of the norepinephrine transporter mRNA in the human placenta and in the JAR placental choriocarcinoma cells was investigated by northern blot analysis using the cDNA of the human (SK-N-SH cell) norepinephrine transporter as the probe (Figure 6). SK-N-SH cells (a human neuroblastoma cell line) which express the norepinephrine transporter but not the dopamine transporter (Richards & Sadee, 1986) were used as a reference. The presence of two mRNAs, 5.8 and 3.6 kb in size, was evident in the human placenta, as was the case in the SK-N-SH cells. However, there was an interesting difference between these two cases. In SK-N-SH cells, the 5.8-kb mRNA species was more predominant than the 3.6-kb mRNA species, confirming earlier findings (Pacholczyk et al., 1991). On the other hand, in the human placenta the 3.6-kb mRNA species was more

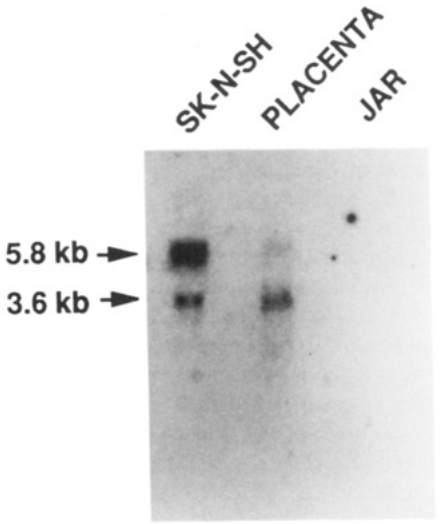

FIGURE 6: Northern hybridization of the human brain norepinephrine transporter cDNA probe with poly(A)$^+$ mRNA isolated from SK-N-SH cells, human placenta, and JAR placental choriocarcinoma cells. RNAs were size-fractionated and detected as described in Materials and Methods. Positions of 5.8- and 3.6-kb hybridizing bands were determined relative to co-blotted RNA standards (Gibco-BRL).

abundant than the 5.8-kb mRNA species. In support of the findings demonstrating the absence of an active norepinephrine uptake mechanism in the JAR cells, there was no mRNA in these cells hybridizing to the cDNA probe.

DISCUSSION

Evidence is provided for the first time in this paper for the presence of a high-affinity active transport system for the norepinephrine in placental brush border membranes. Interestingly, the transport system has higher affinity for dopamine than for norepinephrine and yet its pharmacologic characteristics resemble more closely those of the norepinephrine transporter than those of the dopamine transporter. These two transporters have been well described in the neuronal tissue (Graefe & Bonisch, 1988; Horn, 1990; Iversen, 1975; Kanner & Schuldiner, 1987; Ross, 1987; Trendelenburg, 1991). The neuronal norepinephrine transporter has much higher affinity for dopamine than for norepinephrine (Pacholczyk et al., 1991; Richards & Sadee, 1986; Ritz et al., 1990). Thus, the norepinephrine transporter can effectly transport norepinephrine as well as dopamine. For example, SK-N-SH cells (a neuroblastoma cell line) have the ability to accumulate both dopamine and norepinephrine but possess only the norepinephrine transporter (Richards & Sadee, 1986). Similarly, there is evidence to show that noradrenergic neurons also transport both of these monoamines (Carboni et al., 1990). The dopamine transporter, which is distinct from the norepinephrine transporter, also has higher affinity for dopamine than for norepinephrine (Andersen, 1989; Krueger, 1990; Ritz et al., 1990; Usdin et al., 1991).

Despite the substantial overlap in the specificity for the substrates between the dopamine transporter and the norepinephrine transporter, these two transporters can be distinguished by means of their differential sensitivity to various inhibitors (Giros et al., 1991; Kilty et al., 1991; Pacholczyk et al., 1991; Shimada et al., 1991; Usdin et al., 1991). The human placental brush border membrane vesicles accumulate dopamine (Ramamoorthy et al., 1992b) and norepinephrine (this study). The uptakes of these two monoamines, however, share many common characteristics which include dependence on Na^+ and Cl^-, stimulation by intravesicular H^+ and K^+, and electrogenicity. In addition, the relative rank order potency of various competitive substrates and inhibitors to block the uptake is virtually identical for these two monoamines. These results strongly suggest that the uptake of dopamine and the uptake of norepinephrine occur via a common transporter in human placental brush border membrane vesicles. The pharmacologic characteristics of this uptake process indicate that it is the norepinephrine transporter, and not the dopamine transporter, that is responsible for the uptake. For example, GBR 12909 and bupropion are very weak inhibitors of the uptake of dopamine as well as the uptake of norepinephrine in these membrane vesicles. Comparatively, desipramine and nomifensine are very potent in inhibiting these uptakes. The GBR series of compounds are selective inhibitors of the dopamine transporter (van der Zee et al., 1980), and the most widely used compound in this series to inhibit the dopamine transporter is GBR 12909 (Andersen, 1989). This compound is 3–10 times more potent in inhibiting the dopamine transporter than the norepinephrine transporter (Giros et al., 1991; Pacholczyk et al., 1991; Usdin et al., 1991). On the other hand, desipramine inhibits the norepinephrine transporter with a potency that is at least 3 orders of magnitude greater than its potency to inhibit the dopamine transporter (Giros et al., 1991; Pacholczyk et al., 1991; Usdin et al., 1991). With respect to the uptake of dopamine and norepinephrine, the placental brush border membrane vesicles resemble the human neuroblastoma cell line, SK-N-SH (Richards & Sadee, 1986). In both systems, active transport of the two monoamines occurs via a common transporter. In SK-N-SH cells, the common transporter has been identified as the norepinephrine transporter (Richards & Sadee, 1986), and northern blot analysis with the human norepinephrine transporter cDNA probe confirms the presence of two RNA species, 5.8 kb and 3.6 kb in size, in these cells that hybridize to the probe (Pacholczyk et al., 1991). Two RNA species of comparable size are also detected in the human placenta. The relative abundance of these two RNA species in the human placenta is however clearly different from that in SK-N-SH cells. The JAR cells which do not have the capacity to accumulate dopamine and norepinephrine also do not contain any RNA which hybridizes to the cDNA probe. These data support the conclusion that the human placental syncytiotrophoblast expresses a high-affinity norepinephrine transporter. The basis for two hybridizing RNAs in noradrenergic cells and in the human placenta has yet to be clarified, although similar findings exist for the Na^+/glucose transporter in the human intestine where it is attributable to different lengths of 3′ noncoding region (Hediger et al., 1989).

In addition to establishing the identity of the transport system responsible for the uptake of dopamine and norepinephrine in human placental brush border membrane vesicles as the norepinephrine transporter, the present study also provides some insight into the mechanistic aspects of the transporter. Stoichiometric analysis reveals that one Na^+ and one Cl^- are involved per transport of one norepinephrine molecule. Na^+ and Cl^- might activate the transporter via energetic coupling, i.e., through providing driving force for transport by simultaneous transport down their concentration gradient along with the monoamine substrate. Alternatively, these ions might activate the transporter via catalytic coupling in which case these ions are not translocated across the membrane but stimulate the catalytic activity of the transporter by some other means. It has been shown in the case of many neurotransmitters that Na^+ and Cl^- move across the membrane

coupled to the movement of the neurotransmitter (Kanner & Schuldiner, 1987; Nicholls and Attwell, 1990). Moreover, the data given in Table I show that the activation of norepinephrine transport in placental brush border membrane vesicles by Cl^- is not due anion-induced membrane potentials because no correlation is found between membrane permeability and activation potency for several anions tested. Therefore, it is likely that Na^+ as well Cl^- participate as cotransported ions in the transport of norepinephrine in the placenta. Norepinephrine exists as a cation at pH $\leq$ 7.5. Therefore, if the coupling ratio of Na^+:Cl^-:norepinephrine is 1:1:1 as determined in the present study, the transport process should be electrogenic, provided no other ionic species participates as the cotransportable ion. The present study shows that the NaCl-dependent norepinephrine uptake is electrogenic, and interestingly this characteristic is observed even in the presence of K^+ inside the vesicles. Intravesicular K^+ stimulates the activity of the norepinephrine transporter, but the mechanism of activation is not known. K^+ has a similar stimulatory effect on the serotonin transporter, and the role of K^+ in this case has been investigated in detail in platelet plasma membrane vesicles (Nelson & Rudnick, 1979; Rudnick & Nelson, 1978). The serotonin transporter which operates with a Na^+:Cl^- stoichiometry of 1:1 is electroneutral when K^+ is present inside the vesicles, suggesting that one K^+ is countertransported per transport cycle. The placental serotonin transporter also exhibits similar characteristics (Cool et al., 1990b; Ramamoorthy et al., 1992a). The electrogenic nature of the placental norepinephrine transporter even in the presence of intravesicular K^+ indicates that K^+ is probably not countertransported in the process. The K^+-dependent stimulation of norepinephrine transport is also not due to an inside-negative K^+-diffusion potential as the presence of K^+ inside the vesicles does not enhance the uptake of the lipophilic cation TPP (Table III). Obviously, further work is needed to elucidate the exact role of K^+ in the transport process.

The placental syncytiotrophoblast is a polarized cell, with its brush border membrane in direct contact with the maternal blood. It is this membrane which possesses various active transport systems that are responsible for the transfer of nutrients from mother to fetus (Smith et al., 1992). It is interesting that this membrane also possesses active transport mechanisms for the monoamines. The physiological role of the norepinephrine transporter in this membrane is not known at present. It is possible that the transport system functions to transport norepinephrine from maternal blood into syncytiotrophoblast so as to maintain a low concentration of this monoamine in the intervillous space. The transported monoamine may undergo metabolism inside the cell because the syncytiotrophoblast is known to have high levels of monoamine oxidase A activity (Salach & Detmer, 1979). Such an efficient clearance of norepinephrine from the intervillous space may have physiological importance. Norepinephrine is a well-known activator of smooth muscle contraction. Low levels of norepinephrine in the intervillous space may help to maintain optimal blood circulation in the uteroplacental vascular bed by enabling the uterine arteries which supply the intervillous space to remain open. Such an effect would result in an optimal transfer of nutrients from mother to fetus. Reduced levels of norepinephrine may also prevent uterine contraction and thus lessen the likelihood of premature labor.

Another area in which the current demonstration of the existence of the norepinephrine transporter in the syncytiotrophoblast may become relevant is the maternal abuse of cocaine during pregnancy. The pharmacologic actions of cocaine are mediated by its ability to inhibit the monoamine transporters (Woolverton & Johnson, 1992). The present study clearly shows that the placental norepinephrine transporter is significantly inhibited by cocaine at concentrations (1–2.5 μM) known to be present in the plasma of cocaine users (Javaid et al., 1978; Roe et al., 1990). Complications arising from cocaine abuse during pregnancy include fetal growth retardation, premature birth, stillbirth and increased incidence of abruptio placentae, congenital malformations, vascular disruption, and spontaneous abortion [for a review see Slutsker (1992) and Volpe (1992)]. It is possible that at least some of the fetal and placental complications of cocaine abuse during pregnancy are due to the inhibition of the placental norepinephrine transporter by cocaine and the resultant elevation of norepinephrine in the intervillous space. Currently available theories to explain the toxic effects of cocaine on pregnancy focuses mainly on the pharmacological actions of the drug in the mother, and also on the actions of the drug in the fetus following its transplacental passage (Glantz & Woods, 1991; Volpe, 1992). Our present studies demonstrating the expression of the cocaine-sensitive norepinephrine transporter in the syncytiotrophoblast establish for the first time that the human placenta itself is a target organ for the actions of cocaine.

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