Kinetic Analysis of P-Glycoprotein-Mediated Transport by Using Normal Human Placental Brush-Border Membrane Vesicles

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Purpose. P-Glycoprotein (Pgp) plays an important role in drug disposition and excretion in various tissues such as the brain, intestine, and kidney. Moreover, we have demonstrated that Pgp is expressed on the brush-border membranes of trophoblast cells in the placenta and restricts drug transfer from the maternal circulation to the fetus. However, the transport kinetics of physiologically expressed Pgp has scarcely been investigated.

Methods. In this study, we assessed the functional kinetics of transport mediated by Pgp that is physiologically expressed in normal tissue by using human placental brush-border membrane vesicles (BBMVs). Digoxin and vinblastine were used as typical substrates of Pgp.

Results. The uptakes of [^{3}H]digoxin and [^{3}H]vinblastine into BBMVs were significantly increased in the presence of an ATP-regenerating system. The ATP-dependent uptakes of [^{3}H]digoxin and [^{3}H]vinblastine into BBMVs exhibited saturable kinetics. The Michaelis constants (K_t values) were 2.65 ± 1.80 μM and 21.9 ± 3.37 μM, respectively. In the presence of a Pgp inhibitor such as verapamil, cyclosporine A, or progesterone, the ATP-dependent uptakes of [^{3}H]digoxin and [^{3}H]vinblastine into BBMVs were significantly reduced. Anti-Pgp monoclonal antibody C219 completely inhibited the uptake of [^{3}H]digoxin.

Conclusions. The transport kinetics of [^{3}H]digoxin and [^{3}H]vinblastine by physiologically expressed Pgp were successfully evaluated by using BBMVs prepared from normal human placenta. The present method enabled us to evaluate the function of physiologically expressed Pgp and is superior to the use of cultured transfectants in terms of the yield of vesicles. The present method may also be applicable to investigating the influence of various factors such as the genotype of the *MDR1* gene or various pathophysiologic states of neonates on the function of Pgp.

KEY WORDS: P-glycoprotein; blood–placental barrier; brush-border membrane vesicles; digoxin; vinblastine.

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ABBREVIATIONS: ALP, alkaline phosphatase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; BBMVs, human placental brush-border membrane vesicles; BLMVs, human placental basal membrane vesicles; DHA, dihydroalprenolol; γ-GTP, γ-glutamyltranspeptidase; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; NPPase, nucleotide pyrophosphatase; Pgp, P-glycoprotein.

INTRODUCTION

P-Glycoprotein (Pgp) is encoded by the multidrug resistance gene (*MDR1*) and can confer multidrug resistance by extruding a wide range of structurally unrelated amphiphilic hydrophobic drugs from cells in an ATP-dependent manner (1). Several tissues, such as the brush border of the renal tubule, intestine, liver, and brain capillary endothelium, express Pgp, which extrudes a variety of hydrophobic natural products and drugs a against concentration gradient (2–6). We have already demonstrated that Pgp is localized on the brush-border membrane of human placental trophoblast cells. Moreover, we have also demonstrated by using human placental choriocarcinoma epithelial cells (BeWo) that digoxin, vinblastine, and vincristine are transported unidirectionally from the fetus to the mother by Pgp, possibly to protect the fetus from toxic xenobiotics (7).

Because Pgp is one of the most important factors regulating the pharmacokinetics of several drugs, it is of interest to estimate the transport kinetics of physiologically expressed Pgp. Several studies have been carried out to assess the function of Pgp. Litman *et al.* assessed the function of Pgp in terms of the activity for ATP hydrolysis, using microsomal membranes prepared from Chinese hamster ovary cells (CR1R12) (8,9). Stephens *et al.* and Hunter *et al.* assessed the transport of digoxin and vinblastine, respectively, using Caco-2 monolayers (10,11). However, all these studies were carried with malignant cell lines, and little is known about the function of physiologically expressed Pgp in normal human tissues. It is difficult to obtain a sufficient quantity of a normal human tissue that expresses Pgp for such a study because biopsy of normal tissue, such as colon, is invasive to the donor. Placenta is the only tissue obtainable noninvasively in large amounts. Moreover, trophoblastic membrane vesicles can be readily prepared from the placental tissue. A vesicle study would enable us to investigate separately the function of brush-border and basal membranes under a limited driving force, making it possible to investigate the function of a distinct transporter such as Pgp.

We have already investigated the transport properties of valproic acid and L-lactic acid by using human placental brush-border membrane vesicles (BBMVs) (12). In the present study, in order to establish a method to estimate the transport kinetics of physiologically expressed Pgp in humans, we examined the transport properties of digoxin and vinblastine mediated by Pgp in BBMVs prepared from human placenta.

METHODS

Materials and Reagents

[^{3}H]Digoxin (17.0 Ci/mmol) was purchased from NEN Research Products (Boston, MA). [^{3}H]Vinblastine sulfate (9.4 Ci/mmol) was purchased from Amersham International, plc (Buckinghamshire, UK). Mouse monoclonal antibody C219 (C219) was purchased from TFB Inc. (Tokyo, Japan). Monoclonal anti-β-actin, mouse ascites fluid was purchased from Sigma Chemical Co. (St Louis, MO). Peroxidase-conjugated sheep affinity-purified antibody to mouse IgG was purchased from ICN Pharmaceuticals, Inc. (Aurora, OH).

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Horseradish peroxidase-conjugated rabbit antimouse IgG enhanced chemiluminescence (ECL) kit was purchased from Amersham (Oakville, ON, Canada). Verapamil, digoxin, and 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Cyclosporine A was kindly supplied by Novartis Pharm Inc. (Basel, Switzerland). Vinblastine was kindly supplied by Eli Lilly Japan K.K. (Hyogo, Japan). All other chemicals used in this study were commercial products of reagent grade.

Preparation of Human Placental Brush-Border Membrane Vesicles (BBMVs)

BBMVs were prepared accordingly to the method described by Smith *et al.* (13) with minor modifications. Human term placentas from uncomplicated pregnancies were obtained within 15 min after vaginal or cesarean delivery and placed in 0.9% NaCl at 4°C. After removal of the cord, amniochorion, and decidua, placental tissue was cut from the maternal side and washed in 250 mM mannitol, 10 mM HEPES-Tris at pH 7.4 (MHT buffer). The mince was stirred for 1 h to loosen the microvilli and filtered through two layers of woven cotton gauze. A sample of this starting mince was taken for enzyme analysis. The filtrate was centrifuged at 800 × *g* for 10 min. The pellet was discarded, and $MgCl_2$ was added to the supernatant to a final concentration of 10 mM. After 10 min, with occasional stirring, the supernatant was centrifuged at 10,500 × *g* for 10 min. The pellet was discarded, and the supernatant was centrifuged at 20,000 × *g* for 20 min. The pellet from this run was suspended in MHT buffer with a 25-gauge syringe needle. All the subsequent procedures were performed at 4°C.

Preparation of Human Placental Basal Membrane Vesicles (BLMVs)

Human placental basolateral membrane vesicles (BLMVs) were prepared by the method previously described by Kelley *et al.* (14) with minor modifications. Human term placentas from uncomplicated pregnancies were obtained within 15 min after vaginal or cesarean delivery and placed in 0.9% NaCl at 4°C. After removal of the cord, amniochorion, and decidua, placental tissue was cut from the maternal side and washed in phosphate-buffered saline (PBS(−)). Tissue was stirred in PBS for 30 min and collected on a nylon mesh. The filtrate was washed three times with cold 50 mM Tris-HCl (pH 7.4), collected on a 250-μm pore size nylon mesh, and divided into several equal portions. Each portion was sonicated in 100 ml of the same Tris buffer using a ¾-inch high-gain probe for 10 s at 240 W (Vibra-cell, Sonics and Materials, Newtown, CT). The suspensions were kept on ice. The sonication procedure selectively removes any remaining brush-border membrane. Sonicated tissue was collected on the mesh, washed three times with 5 mM Tris-HCl (pH 7.4), and then stirred gently for 60 min in the same buffer. Tissue was then collected on the nylon mesh and washed again in the same buffer. This procedure disrupts and removes the intracellular components, thus exposing the basolateral membrane. Tissue portions of 25–30 g were resuspended in about 100 ml of 50 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 250 mM sucrose and incubated for 30 min with occasional stirring. Portions were then sonicated twice for 20 s at 250 W to release the basolateral membrane. Suspensions were strained through nylon mesh, and the supernatant was centrifuged at 3,300 × *g* for 10 min to remove debris. The supernatant from this spin was recentrifuged at 80,000 × *g* for 40 min to yield the basolateral membrane pellet, which was resuspended, using a Dounce homogenizer, in 25 mM HEPES-Tris (pH 7.4) containing 1 mM EDTA and 275 mM sucrose. This fraction was further purified by centrifugation on a discontinuous gradient of 10% (w/v) Ficoll (Pharmacia, Peapack, NJ) in the resuspension buffer overlaid with 4% Ficoll [as described by Kelley *et al.* (15)] prepared in 25 mM HEPES-Tris (pH 7.4) containing 1 mM EDTA and 275 mM sucrose. Ficoll gradient tubes were spun at 90,000 × *g* for 1 h. The material at the density gradient interfaces was collected, washed, and resuspended in 25 mM HEPES-Tris (pH 7.4) containing 275 mM sucrose. The suspension from this run was resuspended in the same buffer with a 25-gauge syringe needle. All the operations were carried out at 4°C.

Purity and Orientation of Human Placental Brush-Border Membrane Vesicles (BBMVs) and Human Placental Basal Membrane Vesicles (BLMVs)

To confirm the purity of BBMVs, alkaline phosphatase (ALP) activity and γ-glutamyltranspeptidase (γ-GTP) activity as markers of the brush-border membrane were assayed as reported by Hulstaert *et al.* (16) and Sawabu et al. (37), respectively. The binding activity of [^{3}H]dihydroalprenolol (DHA) as a marker of the basal membrane was assayed as reported by Kelley *et al.* (14). The orientation of membrane vesicles were determined by examining the nucleotide pyrophosphatase (NPPase) (ectoenzyme) activity for hydrolysis of *p*-nitrophenylthymidine 5′-monophosphate in the presence and absence of 1% Triton X as reported by Bohme *et al.* (17). The amount of protein in the cells was measured by Lowry's method (18).

Detection of P-Glycoprotein

P-Glycoprotein was detected by Western blotting analysis. Human myelogeneous leukemia K562 (negative) and adriamycin-resistant K562 (K562/ADM) (positive) were used as control of expression of Pgp (19). All samples were collected, suspended in the lysis buffer containing 100 mM Tris HCl (pH 7.6), 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton, 0.1% sodium dodecyl sulfate (SDS), 0.1% Np-40, 1 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml leupeptin, 0.01 mg/ml aprotinin, and 1 mM sodium vanadate, and incubated for 30–45 min at 4°C. After incubation, the suspension was centrifuged at 15,000 × *g* for 15 min at 4°C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (15). The proteins were transferred electrophoretically onto a 0.2-μm pore size Clear Blot Membrane-P (Atto Corporation, Tokyo, Japan). Blots were blocked overnight at 4°C with 5% nonfat powdered milk in PBS(−). The membranes were incubated with C219 (36) for 1 h at room temperature. The antibody was diluted in PBS(−) containing 1% nonfat powdered milk. Horseradish peroxidase-conjugated rabbit antimouse IgG was used as the secondary antibody. Detection was done with ECL reagents

(Amersham, Oakville, ON, Canada) according to the manufacturer's instructions.

Uptake Studies

The uptakes of [^{3}H]digoxin and [^{3}H]vinblastine into BBMVs were measured by using a rapid filtration technique (20). The ATP-dependent uptake of [^{3}H]digoxin into BBMVs was calculated by subtracting the uptake in the absence from that in the presence of an ATP-regenerating system (21,22). Generally, an aliquot (6 μl) of membrane vesicles (50–60 μg protein) was placed in a microtube, and 9 μl of ATP buffer (10 mM HEPES-Tris, 10 mM $MgCl_2$, 5 mM ATP, 10 mM creatine phosphate, and 100 μg/ml creatine phosphokinase, pH 7.4, plus an appropriate concentration of mannitol to be isotonic) was added. After 30 s, 15 μl of uptake buffer (10 mM HEPES-Tris, pH 7.4, an appropriate concentration of mannitol to be isotonic) containing [^{3}H]digoxin or [^{3}H]vinblastine, with or without unlabeled substrate and inhibitors, was added. ATP-independent association of [^{3}H]digoxin and [^{3}H]vinblastine with the vesicles was determined by replacing ATP with AMP in the ATP buffer. At the designated time at 37°C, the uptake was terminated by the addition of 1 ml of ice-cold buffer (10 mM HEPES-Tris and 260 mM mannitol, pH 7.4). The stopped reaction mixture was filtered through a filter (HAWP 0.45 μm; Millipore Intertech, Bedford, MA) pretreated with 10% albumin to reduce filter binding of the drugs, and then washed twice with 4 ml of ice-cold buffer. Nonspecific binding was determined by dilution of the membrane vesicles with 1 ml of ice-cold buffer before addition of radioactive substrate, followed by filtration. To assay the radiolabeled compounds, filters were put into counting vials, 4 ml of scintillation fluid, Clear-sol I (Nacalai Tesque, Kyoto, Japan), was added, and the radioactivity was determined using a liquid scintillation counter (LC6500, Beckman Instruments, Inc., Fullerton, CA).

Data Analysis

In order to estimate the specific uptake of substrate, correction was made for nonspecific binding. The uptakes of [^{3}H]digoxin and [^{3}H]vinblastine were represented as vesicle/medium ratio (μL/mg protein). Nonspecific binding amounts of [^{3}H]digoxin and [^{3}H]vinblastine were 0.232 ± 0.031 and 2.109 ± 0.040 (μL/mg protein), respectively. To determine the kinetic parameters of [^{3}H]digoxin and [^{3}H]vinblastine uptake into BBMVs, curve fitting to Eq. (1) was done by least-squares nonlinear regression analysis (MULTI; 24).

$$J = J_{max} \times S/(K_t + S) + k_d \times S \quad (1)$$

where J and S represent the transport rate and concentration of substrate, respectively. J_{max}, K_t, and k_d represent the maximum transport rate for saturable transport, the affinity for saturable transport, and the rate constant for nonsaturable transport.

Student's *t* test or ANOVA followed by Duncan's test was used to determine the statistical significance of differences.

RESULTS

Purity and Orientation of BBMVs and BLMVs

In order to confirm the purity of BBMVs, ALP and γ-GTP activities as markers for the brush-border membrane and DHA binding as a marker for the basal membrane were determined. The ALP activity of BBMVs was 36.8-fold and the γ-GTP activity was 22.3-fold higher than those of the homogenate, respectively, and the DHA binding of BBMVs was only 3.72-fold higher than those of the homogenate. On the other hand, the ALP and γ-GTP activities of BLMVs were very much lower than that those of the homogenate, even slightly enriched. Furthermore, DHA binding of BLMVs was 38.9-fold higher than that of the homogenate (Table I). Therefore, the brush-border membranes and the basal membranes were highly purified.

Furthermore, to ascertain the orientation of BBMVs, NPPase activity was determined in the presence or absence of 1% Triton X; the values obtained were 1.03 ± 0.096 and 2.47 ± 0.034 pmol/min/mg protein, respectively, suggesting that 41.9% of BBMVs were inside-out.

Detection of P-Glycoprotein

The expression of Pgp was examined by Western blotting. As shown in Fig. 1, Pgp was detected in BBMVs as well as K562/ADM (positive control) at about 170 kDa but was virtually undetectable in BLMVs or K562 (negative control), using the anti-Pgp mouse monoclonal antibody C219.

ATP-Dependent Uptake of [^{3}H]Digoxin and [^{3}H]Vinblastine into BBMVs

The uptakes of [^{3}H]digoxin and [^{3}H]vinblastine into BBMVs were determined in the presence or absence of an ATP-regenerating system. The uptakes of [^{3}H]digoxin and [^{3}H]vinblastine into BBMVs were significantly stimulated in the presence of this ATP-regenerating system (Fig. 2). As seen in the inset of Fig. 2, the uptakes of [^{3}H]digoxin and [^{3}H]vinblastine were osmolarity dependent, showing that the uptakes contributed little to membrane binding.

Effects of Pgp Inhibitors on the ATP-Dependent Uptake of [^{3}H]Digoxin and [^{3}H]Vinblastine into BBMVs

The uptakes of [^{3}H]digoxin and [^{3}H]vinblastine into BBMVs in the presence of an ATP-regenerating system were significantly inhibited by 20 μM verapamil, 20 μM cyclospor-

Table I. Purity of Human Placental Brush-Border Membrane Vesicles and Basal Membrane Vesicles[a]

	ALP activity (μmol/min/mg protein)	γ-GTP activity (IU/mg protein)	DHA binding (fmol/mg protein)
Brush border			
Homogenate	0.142 ± 0.01	1.77 ± 0.14	3.36 ± 0.62
BBMVs	5.24 ± 0.32	38.9 ± 0.35	12.5 ± 1.86
Enrichment	36.8	22.3	3.72
Basal			
Homogenate	0.789 ± 0.126	1.97 ± 0.42	55.8 ± 5.62
BLMVs	1.47 ± 0.08	6.63 ± 0.95	2173.7 ± 23.2
Enrichment	1.86	3.37	38.9

[a] Alkaline phosphatase (ALP) and γ-glutamyltranspeptidase (γ-GTP) are markers for brush-border membrane. Dihydroalprenolol (DHA) binding is a marker for basal membrane.

Note: Each point represents the mean ± S.E.M. of three experiments.

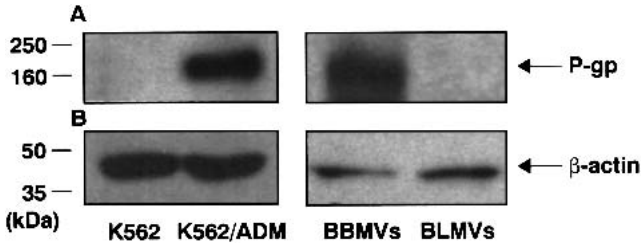

Fig. 1. Immunodetection of P-glycoprotein. Protein samples—K562, 20 μg; K562/ADM, 20 μg; BBMVs, 50 μg; and BLMVs, 50 μg—were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) with a 7.5% polyacrylamide gel and transferred onto Clear Blot Membrane-P (Atto Corporation, Tokyo, Japan). Immunoblots were performed with C219 (A) or an β-actin Mab (B) and developed with the ECL detection reagent, as described in the text.

ine A, and 500 μM progesterone (Fig. 3). The anti-Pgp monoclonal antibody C219 potently inhibited the uptakes of [^{3}H]digoxin and [^{3}H]vinblastine in the presence of the ATP-regenerating system.

Kinetics

The initial uptakes of digoxin and vinblastine into BBMVs in the presence of the ATP-regenerating system were concentration dependent, saturable, and described by Eq. (1) (Fig. 4). The kinetic parameters K_t, J_{max}, and k_d for digoxin were 2.65 ± 1.80 μM, 1.20 ± 0.452 nmol/20 min/mg protein, and 0.193 ± 0.098 μL/20 min/mg protein, respectively (Table II). The kinetic parameters K_t, J_{max}, and k_d for vinblastine were 21.9 ± 3.37 μM, 172.4 ± 37.7 nmol/2 min/mg protein and 4.02 ± 0.128 μL/2 min/mg protein, respectively (Table II).

DISCUSSION

Recently, it has been demonstrated that Pgp is expressed in the normal human intestine, proximal renal tubules, and brain capillary endothelium and plays important roles in the pharmacokinetics and drug–drug interactions of several drugs, including anticancer drugs (2–6). Pgp has also been found in the placenta (3,24), where we have demonstrated that it is localized on the brush-border membrane of human placental trophoblast cells (7). Although Pgp plays an important role in the pharmacokinetics of drugs, little is known about the kinetics of Pgp-mediated transport in normal human tissues. In this study, the kinetic function of Pgp was estimated by using BBMVs prepared from normal human placenta. This is the first study to investigate in a quantitative and kinetic manner the function of physiologically expressed Pgp.

The purity of BBMVs was assessed in terms of the ALP and γ-GTP activities as markers of the brush-border membrane and DHA binding as a marker of the basal membrane. In comparison with homogenate, the ALP and γ-GTP activities of BBMVs were increased 36.8- and 22.3-fold, respectively, but the DHA binding of BBMVs was only 3.72 times that of the homogenate. Furthermore, Pgp was detected on the brush-border membranes of K562/ADM cells but not on the basal membranes or in the parental K562 cells. Pgp is known to be a marker of placental brush-border membrane (7,25,26). Therefore, BBMVs were considered to have been highly purified in this study. The NPPase assay revealed that 41.9% of BBMVs were inside-out. Therefore, the function of Pgp can be estimated by an uptake study using these BBMVs in the presence of the ATP-regenerating system.

The uptakes of [^{3}H]digoxin and [^{3}H]vinblastine into

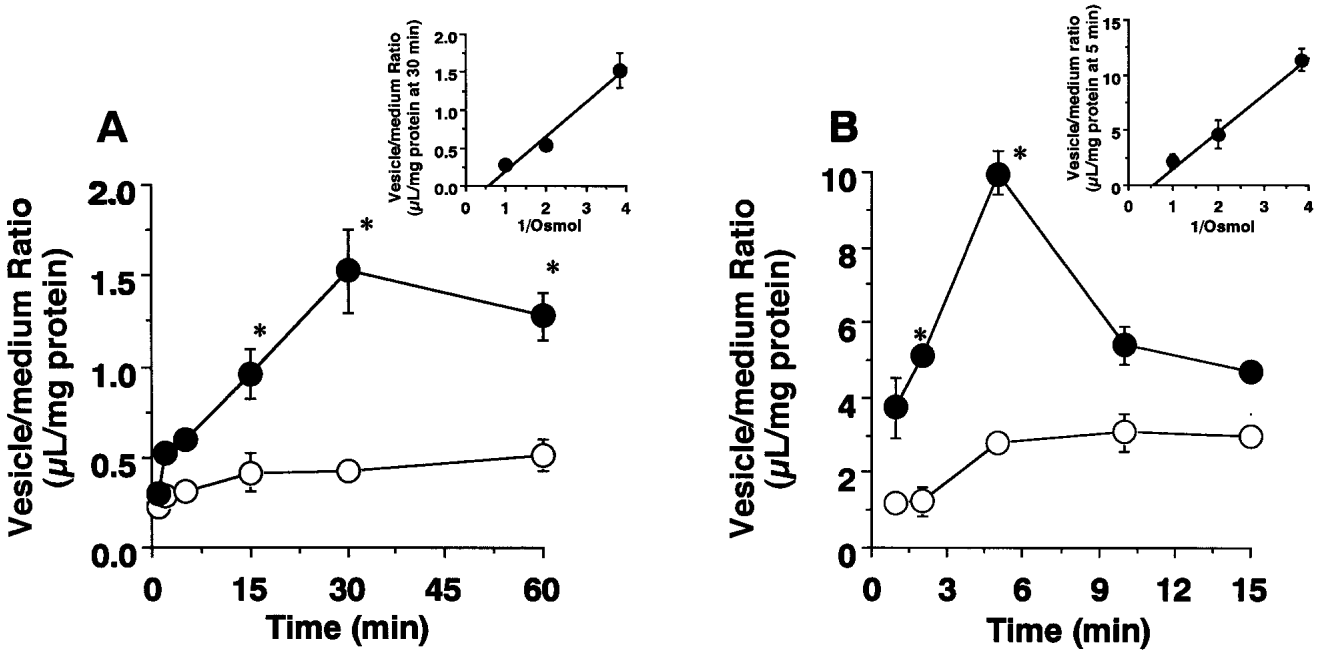

Fig. 2. ATP-dependent uptake of 50 nM [^{3}H]digoxin and 50 nM [^{3}H]vinblastine into BBMVs. The uptakes of [^{3}H]digoxin (A) and [^{3}H]vinblastine (B) into BBMVs were measured in the presence *(closed circles)* or absence *(open circles)* of an ATP-regenerating system. **Inset:** Effect of osmolarity on the uptake of [^{3}H]digoxin and [^{3}H]vinblastine into BBMVs. Each point represents the mean ± S.E.M. of four experiments. The significance of differences from the uptake in the absence of the ATP-regenerating system were determined by using Student's *t* test (*$p < 0.05$).

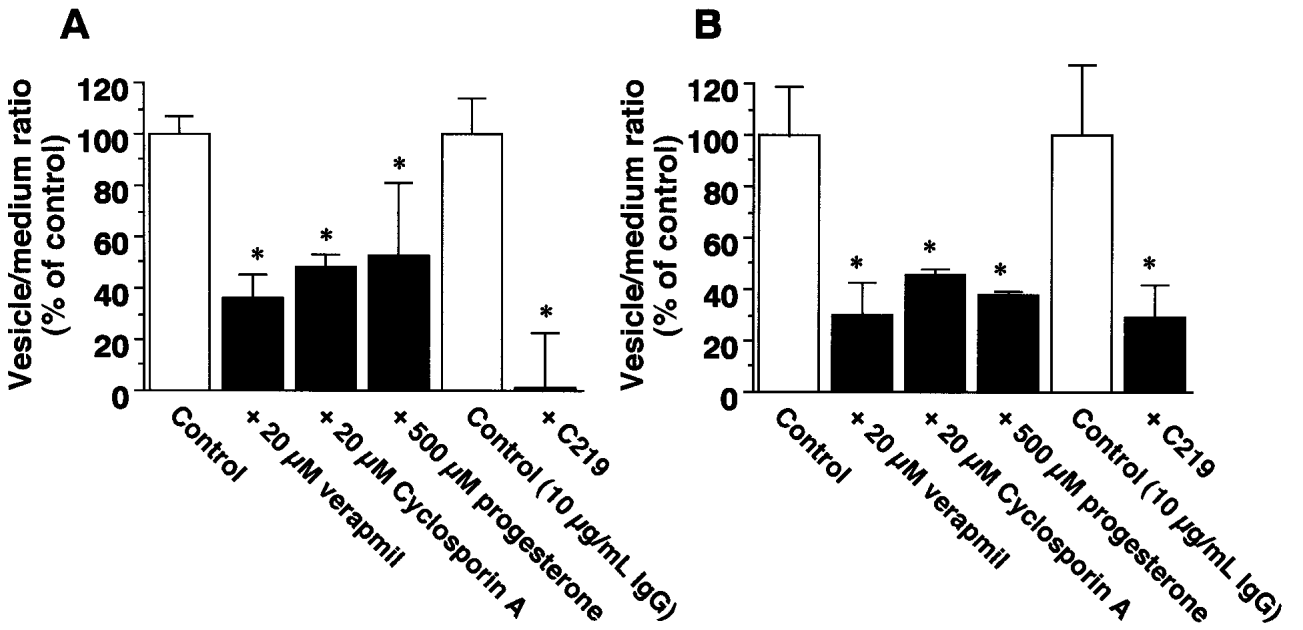

Fig. 3. Effect of inhibitors on the ATP-dependent component of the uptake of 50 nM [^{3}H]digoxin and 50 nM [^{3}H]vinblastine into BBMVs. The ATP-dependent uptake of [^{3}H]digoxin (A) and [^{3}H]vinblastine (B) in the presence *(closed columns)* or absence [Control; 1.01 ± 0.069 μL/mg protein at 30 min (digoxin), 4.72 ± 0.922 μL/mg protein at 5 min (vinblastine), 10 μg/mL IgG; 0.743 ± 0.103 (digoxin), 3.57 ± 1.66 (vinblastine), *open columns*] of 20 μM verapamil, 20 μM cyclosporine A, 500 μM progesterone, and C219 anti-Pgp monoclonal antibody. Each point represents the mean ± S.E.M. of four experiments. The ATP-dependent uptake calculated by subtracting values obtained in the absence of the ATP-regenerating system from values obtained in the presence of the ATP-regenerating system. The significance of differences from the control were determined by using ANOVA followed by Duncan's test (*$p < 0.05$).

BBMVs were significantly increased in the presence of the ATP-regenerating system (Fig. 2) and were significantly inhibited by typical Pgp inhibitors and anti-Pgp monoclonal antibody C219 (Fig. 3). Anti-Pgp monoclonal antibody C219 completely inhibited the uptake of [^{3}H]digoxin but inhibited only 65% of the uptake of [^{3}H]vinblastine. Therefore, the uptake of digoxin in the presence of the ATP-regenerating system is essentially wholly attributable to Pgp, whereas the

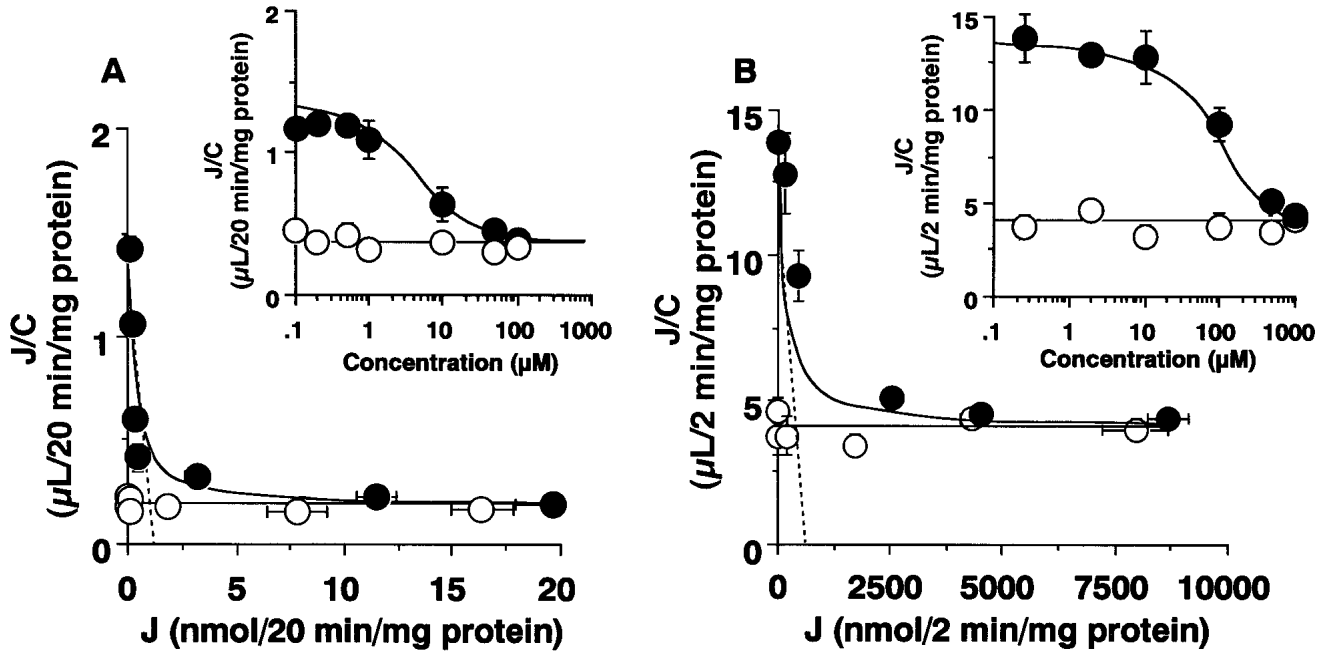

Fig. 4. The concentration-dependent uptake of digoxin and vinblastine into BBMVs. Eadie–Hofstee plot of digoxin (A) and vinblastine (B) in the presence *(closed circles)* or absence *(open circles)* of the ATP-regenerating system at 20 min and 2 min, respectively. The concentrations of digoxin and vinblastine were 100, 200, 500 nM, 1, 10, 50, 100 μM; and 250 nM, 2, 10, 100, 500 μM, 1 mM; respectively. The *lines* represent the calculated curves obtained by fitting to Eq. (1). Saturable components of digoxin and vinblastine are represented by *dotted lines.* Each point represents the mean ± S.E.M. of four experiments.

Table II. Kinetic Parameters for the Uptakes of Digoxin and Vinblastine into Brush-Border Membrane Vesicles in the Presence of the ATP-Regenerating System[a]

Substrate	J_{max}	K_t(μM)	k_d
Digoxin	1.20 ± 0.452 (nmol/20 min/mg protein)	2.65 ± 1.80	0.193 ± 0.098 (μL/20 min/mg protein)
Vinblastine	172.4 ± 37.7 (nmol/20 min/mg protein)	21.9 ± 3.37	4.02 ± 0.128 (μL/20 min/mg protein)

[a] Estimated value ± S.D. of the estimated residual weighted sum of squares.

uptake of vinblastine may be partially attributed to some other ATP-dependent transport system. Thus, digoxin may be a more suitable substrate than vinblastine to estimate the function of Pgp.

So far, several investigators have estimated the kinetics function of Pgp by using human malignant cell lines, vesicles prepared from transfectants, and flow cytometry of $CD56^+$ natural killer cells obtained from humans. Stephens *et al.* (11) demonstrated, using Caco-2 (a human malignant cell line) monolayers, that the ED_{50} values of basal-to-apical transcellular transport of digoxin and vinblastine were 58.2 μM and 26.5 μM, respectively. They also showed, using an Ussing chamber, that the ED_{50} values of digoxin transport across rat jejunum, ileum, and colon and human colon membranes were 81.4, 74.3, 50.6, and 58.7 μM, respectively, and the ED_{50} value of vinblastine across the rat ileum was 48.2 μM (11). Similarly, Hunter *et al.* (10) reported that the K_m value of basal-to-apical transcellular transport of vinblastine across a Caco-2 monolayer was 18.99 μM. Horio *et al.* (21) investigated the ATP-dependent uptake of vinblastine into vesicles prepared from multidrug-resistant human KB carcinoma cells (KB-V1) and reported a K_m value of 2 μM. The K_m value of vinblastine obtained by determining the ATPase activity of Pgp expressed in the Chinese hamster ovary cell line CHO CR1R12 was 1.3 μM (8,9). In our present study, kinetic analysis revealed that the K_t values of digoxin and vinblastine were 2.65 and 21.9 μM (Table II), respectively. Especially in the case of digoxin, ED_{50} or K_m values in the literature are considerably higher than the K_t values in this study. However, the ED_{50} values obtained from transport studies may be influenced by several factors, such as the contributions of other transporters, passive diffusion, intracellular distribution, and so on. In the vesicle studies, the K_m value for vinblastine in KB-V1 vesicles was 10-fold lower than the K_t value for vinblastine in this study, suggesting that the function of Pgp in malignant cell lines may be different from that of Pgp expressed under the physiologic condition. We consider that the present method using BBMVs is the most suitable to estimate the K_t values of various drugs mediated by a physiologically expressed transporter.

With respect to the parameter, J_{max}, it is highly dependent on the amount of Pgp in BBMVs. Stephens *et al.* (11) reported that the J_{max} value of basal-to-apical digoxin transport across a Caco-2 monolayer was 13.0 nmol/h/cm^2, which is 2.3-fold higher than that of vinblastine. On the other hand, the J_{max} value of digoxin into BBMVs was 1.20 nmol/2 min/mg protein, which is less than 1% of that for vinblastine found in this study. Although the reason for the discrepancy remains unclear, several factors may have complex effects on the parameter in a study using cells, as in the case of the K_t value. The relative J_{max} value obtained in this study may properly reflect the functional activity of Pgp under the physiologic condition. Moreover, quantitative determination of Pgp in BBMVs may enable us to evaluate the maximum transport activity for each drug per molecule of Pgp.

Recently, several polymorphisms have been identified in the *MDR1* gene encoding Pgp. Hoffmeyer *et al.* (27) reported that a single nucleotide polymorphism (SNP) in exon 26 (C3435T) of *MDR1*, without amino acid substitution, correlates with the level of Pgp in the intestine and the oral availability of digoxin. Moreover, several SNPs with amino acid substitution have been reported in the *MDR1* gene (26–33). Therefore, it is important to determine the influence of the above SNPs on the transport kinetics of Pgp substrates. Kim *et al.* (34) reported that the SNP G2677T ($Ala^{893}Ser$) results in decreased cellular accumulation of digoxin without affecting the expression level of Pgp. However, little is known about the relationship between genotype and phenotype of Pgp as regards the kinetics in normal human tissues. In one study, Hitzl *et al.* (35) demonstrated, by using flow cytometry of $CD56^+$ natural killer cells obtained from healthy volunteers, that the function of Pgp with the SNP C3435T is less than that of wild-type Pgp, with rhodamine 123 as a substrate. However, they could not establish whether this resulted from a change in the expression level, the transport rate, or the affinity of Pgp. The present method enabled us to determine the kinetic parameters of the uptakes of digoxin and vinblastine mediated by Pgp in an ATP-dependent manner. We could obtain sufficient membrane fraction to estimate the transport kinetics and expression level of Pgp from a genotyped human tissue. Moreover, the present method may also be applicable to an investigation of the influence of various factors, such as pathophysiologic states of the neonate, on the function of Pgp.

In conclusion, the present method using BBMVs of human placenta enabled us to estimate the kinetics of physiologically expressed Pgp in a quantitative manner. It should be applicable to investigating the influence of various factors, such as the genotype of the *MDR1* gene or the pathophysiologic state of the neonate, on the function of Pgp.

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