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Breast Cancer Resistance Protein Exports Sulfated Estrogens but Not Free Estrogens

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ABSTRACT

Breast cancer resistance protein (BCRP), an ATP-binding cassette transporter, confers resistance to a series of anticancer reagents such as mitoxantrone, 7-ethyl-10-hydroxycamptothecin, and topotecan. We reported previously that estrone and 17β-estradiol reverse BCRP-mediated multidrug resistance. In the present study, we demonstrate that BCRP exports estrogen metabolites. First, we generated *BCRP*-transduced LLC-PK1 (LLC/BCRP) cells, in which exogenous BCRP is expressed in the apical membrane, and investigated transcellular transport of ^{3}H-labeled compounds using cells plated on microporous filter membranes. The basal-to-apical transport (excretion) of mitoxantrone, estrone, and 17β-estradiol was greater in LLC/BCRP cells than in LLC-PK1 cells. Thin-layer chromatography of transported steroids revealed that the transport of estrone and 17β-estradiol was independent of BCRP expression. Alternatively, increased excretion of estrone sulfate and 17β-estradiol sulfate was observed in LLC/BCRP cells. BCRP inhibitors completely inhibited the increased excretion of sulfated estrogens across the apical membrane. Conversion of estrogens into their sulfate conjugates was similar between LLC/BCRP and LLC-PK1 cells, suggesting that the increased excretion of estrogen sulfates was attributable to BCRP-mediated transport. Next, the uptake of ^{3}H-labeled compounds in membrane vesicles from *BCRP*-transduced K562 (K562/BCRP) cells was investigated. ^{3}H-labeled estrone sulfate, but not ^{3}H-labeled estrone or 17β-estradiol, was taken up by membrane vesicles from K562/BCRP cells, and this was ATP-dependent. Additionally, BCRP inhibitors suppressed the transport of estrone sulfate in membrane vesicles from K562/BCRP cells. These results suggest that BCRP does not transport either free estrone or 17β-estradiol but exports sulfate conjugates of these estrogens.

Breast cancer resistance protein (BCRP) is a member of the ATP-binding cassette transporter G family and is also known as ABCG2 or ABCP. BCRP mediates concurrent resistance to such chemotherapeutic agents as mitoxantrone (MXR), SN-38 (an active metabolite of CPT-11), and topotecan, presumably by pumping these reagents out of the cell, thereby resulting in concentrations lower than cytotoxic levels (Allikmets et al., 1998; Doyle et al., 1998; Maliepaard et al., 1999; Miyake et al., 1999; Kawabata et al., 2001).

Although the function of BCRP as a drug transporter has been intensively investigated, there is still much to be elucidated concerning its physiological role. BCRP is normally expressed in a wide variety of organs such as placenta, intestine, liver, ovary, testis, kidney, brain and also in hematopoietic stem cells (Allikmets et al., 1998; Doyle et al., 1998; Zhou et al., 2001). In particular, BCRP is highly expressed in the syncytiotrophoblasts of the placenta (Maliepaard et al., 2001). In our previous screening for chemicals that circumvent BCRP-mediated drug resistance, estrone (E_1) and 17β-estradiol (E_2) were found to effectively restore drug sensitivity in *BCRP*-transduced K562 (K562/BCRP) cells (Imai et al., 2002). In light of the function of the syncytiotrophoblasts that synthesize and secrete these estrogens in a mother's body, E_1 and E_2 are therefore candidates as physiological substrates of BCRP.

The present study was designed to examine the direct transport of both E_1 and E_2. First, we performed a transcellular transport assay by using *BCRP*-transfected LLC-PK1 cells, which form a highly polarized monolayer in either a culture dish or on a membrane filter (Ueda et al., 1992; Evers et al., 1996; Jonker et al., 2000). Exogenous BCRP is expressed in the apical membrane of the *BCRP*-transfected

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ABBREVIATIONS: BCRP, breast cancer resistance protein; SN-38, 7-ethyl-10-hydroxycamptothecin; LLC/BCRP, *BCRP*-transduced LLC-PK1; MXR, mitoxantrone; E_1, estrone; E_2, 17β-estradiol; K562/BCRP, *BCRP*-transduced K562; E_1S, estrone 3-sulfate; E_2S, 17β-estradiol 3-sulfate; $E_2$17G, 17β-estradiol 17-glucuronide; TLC, thin-layer chromatography; LLC/EGFP-BCRP, *EGFP-BCRP*–transfected LLC-PK1; EGFP, enhanced green fluorescence protein; FTC, fumitremorgin C; VRP, verapamil; P-gp, P-glycoprotein.

LLC-PK1 cells (Jonker et al., 2000). The BCRP-mediated transcellular transport of ^{3}H-labeled MXR and specific steroids was investigated. The effect of specific compounds on MXR or estrogen transport by BCRP was also examined. Next, the uptake of ^{3}H-labeled compounds in membrane vesicles from K562/BCRP cells was investigated, as was the effect of compounds on estrone 3-sulfate (E_1S) transport by BCRP. The results from these experiments have provided information that will help in our understanding of the physiological functions of BCRP, in addition to its interaction with steroids and other compounds.

Materials and Methods

Reagents. [^{3}H]E_1 (94 Ci/mmol), [^{3}H]E_2 (99 Ci/mmol), and [^{3}H]cortisol (64 Ci/mmol) were purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). [^{14}C]Inulin (2.4 mCi/g), [^{3}H]E_1S (43.5 Ci/mmol), and [^{3}H]17β-estradiol 17-glucuronide ($E_2$17G) (40.5 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). [^{3}H]MXR (3 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO).

Establishment of LLC/BCRP Cells. LLC-PK1 cells, the epithelial cells of the porcine kidney, were cultured in M199 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. LLC-PK1 cells were transduced with an HaBCRP retrovirus supernatant (Imai et al., 2002). Cells were then treated with increasing doses of MXR (2, 4, and 8 nM) for 17 days to enrich the transduced cells. The resulting mixed population of MXR-resistant cells, LLC/BCRP, was used in this study. Expression of BCRP was confirmed by Western blot analysis with the anti-BCRP polyclonal antibody 3488, as described previously (Kage et al., 2002). Anticancer drug resistance levels of BCRP-expressing cells were evaluated by the examination of cell-growth inhibition after incubation at 37°C for 5 days in the presence of various concentrations of anticancer drugs. In addition, the effects of BCRP inhibitors on MXR sensitivity were investigated. Cell numbers were determined with a Coulter counter (Beckman Coulter, Inc., Fullerton, CA). The IC_{50} values were determined from growth inhibition curves.

Transcellular Transport Assay. Exponentially growing cells were plated on 3-μm pore Transwell 3414 filters (Costar, Cambridge, MA) at a density of 2.4×10^6 cells/well and cultured for 3 days. Culture medium in both the upper and lower wells was replaced with 2 ml of serum-free M199 medium 1.5 h before the experiments. The medium in either the upper or lower well was then replaced with 2 ml of medium containing ^{3}H-labeled compounds and/or ^{14}C-labeled inulin. The cells were incubated at 37°C in 5% CO_2, and 50 μl of the medium from the opposite side was sampled after 1, 2, and 4 h from the addition of ^{3}H-labeled compounds and/or ^{14}C-labeled inulin. Radioactivity of each sample was measured by liquid scintillation counting and presented as a percentage fraction of the total radioactivity before incubation. All data were presented as mean values with standard deviations of triplicate determinations from three different cultures. The inhibitory effect of the compounds on the transcellular transport of either ^{3}H-labeled MXR or steroids was examined by adding the compounds to both wells 1.5 h before beginning the experiments. The subsequent procedure was as described above.

Ether Extraction and Silica-Gel Thin-Layer Chromatography of Transported Steroids. Transported ^{3}H-labeled steroids were analyzed by ether extraction and silica-gel TLC. The experimental procedure was the same as that of the transepithelial transport assay until sample collection. At 0.5 or 1 h after the addition of [^{3}H]E_1 or [^{3}H]E_2, all 2 ml of medium in the opposite side was collected, and the total radioactivity was determined from 50 μl of each sample. Thereafter, free estrogens were separated from estrogen metabolites by diethyl ether. In this condition, free estrogens are extracted to the organic phase, and sulfate or glucuronide conjugates of estrogens remain in the aqueous phase (Mellor and Hobkirk, 1975). Radioactivities in the organic fraction (free estrogens) and the aqueous fraction (estrogen metabolites) were then measured. The total radioactivity before the ether extraction and the radioactivity in the aqueous fraction of transported ^{3}H-labeled estrogens are presented as percentage fractions of the radioactivity before the incubation.

For silica-gel TLC, the experimental procedure was the same as that of the transepithelial transport assay until sample collection. A 50-μl sample of the medium in the apical side after 1- and 2-h incubations was mixed with 100 μl of methanol, spotted, and run simultaneously with standard unlabeled estrogens and derivatives (100 μg each) on silica-gel 60 F_{254} plates (Merck, Darmstadt, Germany) in chloroform/methanol/acetic acid (8:3:1). Separated zones, visualized under ultraviolet, were cut, and their radioactivities were determined. All data were presented as mean values with standard deviations of triplicate determinations.

Subcellular Localization of Exogenous BCRP in *EGFP-BCRP*–transfected LLC-PK1 Cells. *BCRP* cDNA was inserted into a pEGFP-C1 vector plasmid (BD Biosciences CLONTECH, Palo Alto, CA). LLC-PK1 cells were transfected with the resulting *EGFP-BCRP* expression construct and selected by exposure to 8 nM MXR for 14 days. Hundreds of drug-resistant colonies, mixed cultures of stable transformants, were pooled and designated as LLC/EGFP-BCRP cells. Expressions of enhanced green fluorescence protein (EGFP)-BCRP fusion protein, anticancer drug resistance, and transporting activity of ^{3}H-labeled compounds were evaluated as described above. Subcellular localization of exogenous BCRP was examined in sagittally cut sections of LLC/EGFP-BCRP cells cultured on the microporous filter membranes.

Preparation of Membrane Vesicles from K562/BCRP Cells. Establishment and characterization of K562/BCRP cells were described previously (Imai et al., 2002). Membrane vesicles were prepared according to the method described previously (Naito et al., 1988). Briefly, K562 or K562/BCRP cells (2×10^9) were suspended in 32 ml of hypotonic buffer [10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM 4-(2-aminoethyl)-benzensulfonyl fluoride, and 20 μg/ml aprotinin] and homogenized using a Dounce homogenizer. The homogenate was centrifuged at 200*g* for 10 min. The supernatant was overlaid on 35% sucrose solution buffered with 10 mM Tris-HCl, pH 7.4, and centrifuged at 45,000*g* for 60 min. The membrane fraction at the interface was collected and precipitated by centrifugation at 138,000*g* for 60 min. The pellet (membrane vesicles) was resuspended in 10 mM Tris-HCl, pH 7.4, and 250 mM sucrose. The membrane vesicles were diluted to protein concentrations of 2 or 5 mg/ml, aliquoted, and stored at −80°C until use.

Intravesicular Transport Assay. The intravesicular transport assay was performed by a rapid centrifugation technique. Vesicles were thawed quickly at 37°C and kept on ice. The transport reaction mixture (50 μl) contained 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 250 mM sucrose, 10 mM phosphocreatine, and 100 μg/ml creatine phosphokinase, with or without 5 mM ATP, radiolabeled and unlabeled compounds, and membrane vesicles (50 μg of protein). The amount of membrane vesicles was increased to 200 μg/reaction in the [^{3}H]$E_2$17G uptake experiment. First, the transport reaction mixture was kept on ice for 5 min. Then the reaction tube was incubated at 37°C for an appropriate time. The reaction was terminated by placing the tube on ice and adding 1 ml of ice-cold stop solution (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 250 mM sucrose). The membrane vesicles were precipitated by centrifugation at 18,000*g* for 10 min at 4°C. The pellet was solubilized with 100 μl of 0.1 M NaOH and then neutralized by the addition of 0.1 M HCl. The radioactivity was counted with the use of a liquid scintillation counter.

Statistical Analysis. The two-sided unpaired Student's *t* test was used to evaluate the statistical significance of the differences between the two sets of data. The difference was considered significant when the *p* value was less than 0.05.

Results

Establishment of LLC/BCRP Cells. BCRP expression in LLC/BCRP cells, a mixed culture of stable transformants, was examined by Western blot analysis in the presence of reducing agents. Under these conditions, BCRP was detected as a monomer of 70 kDa (Fig. 1A). The BCRP expression level in LLC/BCRP cells was stable for at least 2 months without any further drug selection. LLC/BCRP cells were five to six times more resistant to MXR and SN-38 than were the parental LLC-PK1 cells (Fig. 1B). Fumitremorgin C (FTC), an inhibitor of BCRP, and E_1 reversed the MXR resistance of LLC/BCRP cells (Fig. 1B) (Rabindran et al., 2000), which were as sensitive as LLC-PK1 cells in the presence of 3 μM FTC. E_1 (10 μM) circumvented the MXR resistance of LLC/BCRP cells, although LLC/BCRP cells remained more resistant than LLC-PK1 cells. E_2 also showed similar effects on the MXR resistance of LLC/BCRP cells (data not shown). These growth-inhibition assays suggested that the parental LLC-PK1 cells expressed endogenous MXR transporters that were sensitive to FTC and E_1. However, exogenous BCRP expression in LLC/BCRP cells conferred far stronger anticancer drug resistance than did the endogenous transporters, which can also be suppressed by FTC and E_1.

Polarized Transport of [^{3}H]MXR in LLC/BCRP Cells. The transcellular transport of [^{3}H]MXR was examined using a fixed MXR concentration of 50 nM. The paracellular fluxes monitored by [^{14}C]inulin appearance in the other side were less than 1% of the total radioactivity per hour (Fig. 2A). The IC_{50} values of MXR for LLC-PK1 and LLC/BCRP cells in a 5-day treatment were 0.58 and 2.7 nM, respectively (Fig. 1B). However, treatment of the cells with 50 nM MXR for 4 h did not result in [^{14}C]inulin leakage, suggesting that the cells maintained a monolayer structure during the experimental

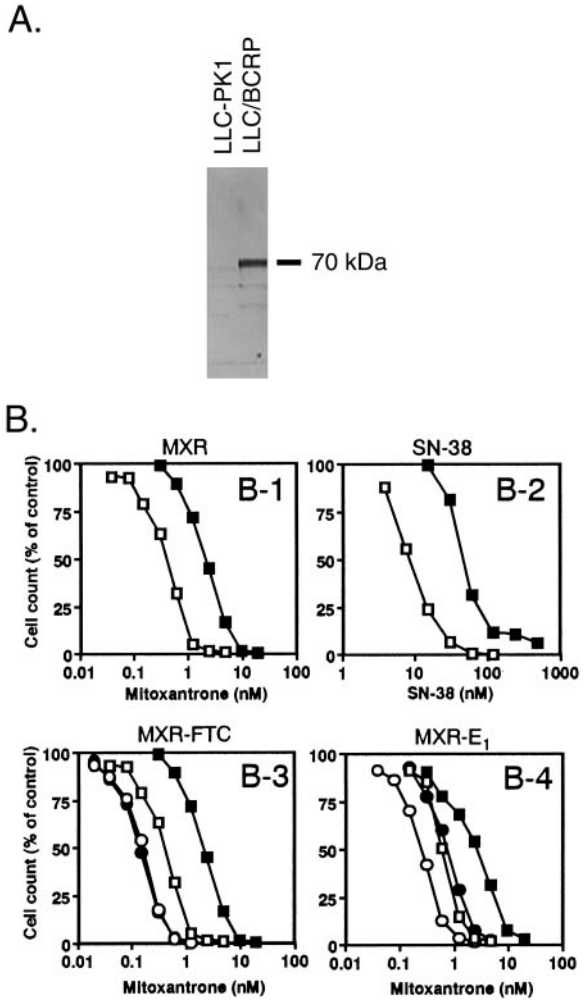

Fig. 1. Characterization of LLC/BCRP cells. A, Western blot analysis of BCRP. Western blotting with the anti-BCRP polyclonal antibody 3488 detected BCRP as a 70-kDa band. B, drug sensitivity test. Cells were incubated at 37°C for 5 days in the presence of increasing concentrations of the indicated anticancer drug with or without modifying agents. Cell numbers were determined with use of a Coulter counter. B-1, sensitivity to MXR. B-2, sensitivity to SN-38. B-3, reversal effect of 3 μM FTC on MXR resistance. B-4, reversal effect of 10 μM E_1 on MXR resistance. □, LLC-PK1 cells without modifying agents; ■, LLC/BCRP cells without modifying agents; ○, LLC-PK1 cells with modifying agents; ●, LLC/BCRP cells with modifying agents. The data are means of triplicate determinations. Error bars are within the symbols.

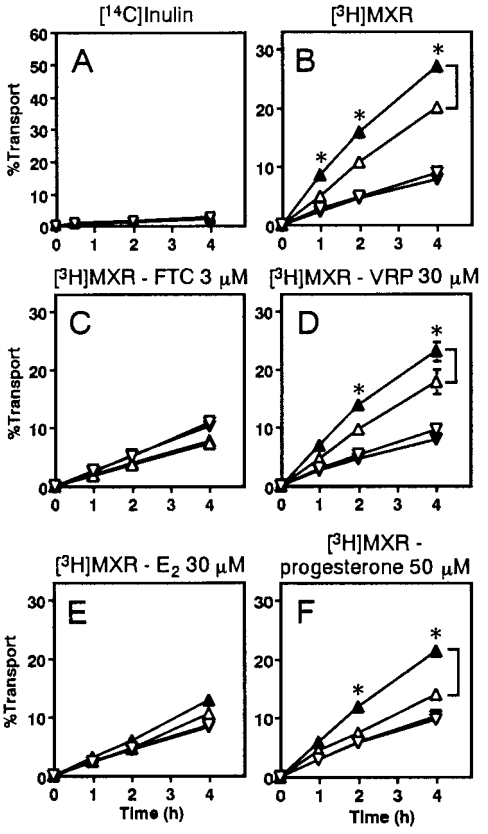

Fig. 2. Transcellular transport of [^{3}H]MXR. Cells (2.4×10^6 /well) were plated on 3-μm pore filters 3 days before beginning the experiment. When needed, modifying agents were added to the medium in both the apical and basal side 1.5 h before the experiment. [^{14}C]inulin and/or [^{3}H]MXR was added to either the apical or the basal side of the medium. After 1, 2 and 4 h, the percentage of radioactivity that appeared in the opposite side was measured. A, paracellular efflux of 2.1 ng/ml [^{14}C]inulin in the presence of 50 unlabeled MXR. B, transport of 50 nM [^{3}H]MXR in the absence of modifying agents. C, transport of 50 nM [^{3}H]MXR in the presence of 3 μM FTC. D, transport of 50 nM [^{3}H]MXR in the presence of 30 μM VRP. E, transport of 50 nM [^{3}H]MXR in the presence of 30 μM E_2. F, transport of 50 nM [^{3}H]MXR in the presence of 50 μM progesterone. △, excretion (basal-to-apical transport) in LLC-PK1; ▲, excretion in LLC/BCRP; ▽, reabsorption (apical-to-basal transport) in LLC-PK1; ▼, reabsorption in LLC/BCRP. The data, presented as a percentage fraction of the total radioactivity, are means ± S.D. of triplicate determinations from three different cultures. Where a vertical bar is not shown, standard deviation is within the symbol. *, $p < 0.05$ in the excretion. These results were confirmed at least three times.

period (Fig. 2A). The basal-to-apical [^{3}H]MXR transport (excretion) in LLC/BCRP cells was greater than that in LLC-PK1 cells, whereas the apical-to-basal [^{3}H]MXR transport (reabsorption) in LLC/BCRP cells was similar to that in LLC-PK1 cells (Fig. 2B). FTC (3 μM) completely abolished the increased excretion of [^{3}H]MXR in LLC/BCRP cells, so that [^{3}H]MXR excretions in both cell lines were almost identical in the presence of FTC (Fig. 2C). FTC did not affect the [^{3}H]MXR reabsorption. Verapamil (VRP) (30 μM), an inhibitor of P-glycoprotein (P-gp) and MRP1, did not suppress the increased excretion of [^{3}H]MXR in LLC/BCRP cells compared with LLC-PK1 cells (Fig. 2D) (Tsuruo et al., 1981; Cole et al., 1994). VRP also did not affect the reabsorption of [^{3}H]MXR. These results suggest that the increased excretion of [^{3}H]MXR in LLC/BCRP is mediated by exogenous BCRP expressed in the apical membrane of the cells.

We demonstrated previously that BCRP-mediated drug resistance was overcome by E_1 and E_2 (Imai et al., 2002). The inhibitory effect of steroids on BCRP-mediated drug transport was validated by a transcellular transport assay of [^{3}H]MXR. LLC/BCRP and LLC-PK1 cells showed similar sensitivity to E_1, E_2, or progesterone. The IC_{50} values of E_1, E_2, and progesterone in a 5-day treatment were approximately 22, 10, and 12 μM, respectively. Transcellular transport experiments using [^{3}H]MXR (50 nM) with steroids (up to 50 μM) did not result in [^{14}C]inulin leakage, suggesting that the cells maintained a monolayer structure during the experimental period of 4 h (data not shown). E_2 (30 μM) strongly suppressed the BCRP-dependent increase in MXR excretion (Fig. 2E). E_1 also suppressed the BCRP-dependent increase in MXR excretion (data not shown). The BCRP-dependent increase in MXR excretion was not affected by progesterone (50 μM), in accordance with our previous result that progesterone was unable to surmount BCRP-mediated drug resistance (Fig. 2F) (Imai et al., 2002).

Transcellular Transport of Steroids in LLC/BCRP Cells. Because our previous study raised the possibility that E_1 and E_2 were in fact substrates of BCRP, the transcellular transport of ^{3}H-labeled estrogens was investigated. [^{3}H]E_1 excretion in LLC/BCRP cells was greater than that in LLC-PK1 cells, whereas its reabsorption in LLC/BCRP cells was reduced compared with that in LLC-PK1 cells (Fig. 3A). Similarly, LLC/BCRP cells showed higher [^{3}H]E_2 excretion and less [^{3}H]E_2 reabsorption than did LLC-PK1 cells (Fig. 3B). In the transport experiments using [^{3}H]E_1 and [^{3}H]E_2, excretion and reabsorption were similar in LLC/BCRP cells at an earlier time point (1 h), but excretion exceeded reabsorption at the later time points (2 and 4 h). Exogenous BCRP expression had no effect on the transcellular transport of [^{3}H]cortisol and [^{3}H]E_1S (Fig. 3, C and D). Excretion and reabsorption of [^{3}H]E_1S were equally low in LLC/BCRP and LLC-PK1 cells. Exogenous BCRP also had no effect on the transcellular transport of [^{3}H] progesterone (data not shown).

Fractionation of Transported Steroids by Ether Extraction. The greater differences in the measured excretion of [^{3}H]E_1 and [^{3}H]E_2 at the later time points (2 and 4 h) suggested that there was metabolic modification of these steroids. To examine this possibility, radioactive compounds that appeared on the other side after a 0.5- or 1-h incubation were extracted with diethyl ether. In this analysis, 99% of free estrogen was extracted in the organic fraction, and 99% of conjugated estrogens remained in the aqueous fraction. The ether-nonextractable radioactivity in the excretion medium of LLC/BCRP cells was higher than that of LLC-PK1 cells (Fig. 4A). The increased excretion of the ether-nonextractable ^{3}H-labeled compounds in LLC/BCRP cells was suppressed in the presence of 3 μM FTC, so that excretion of the ether-nonextractable ^{3}H-labeled compounds became identical between LLC/BCRP and LLC-PK1 (Fig. 4A). Additionally, the ether-nonextractable radioactivity in the reabsorption medium of LLC/BCRP cells was lower than that in LLC-PK1 cells (Fig. 4B). In contrast, there was no significant difference in the ether-extractable radioactivity in both excretion and reabsorption between LLC/BCRP and LLC-PK1, and FTC did not affect excretion of the ether-extractable fraction in both cell lines (data not shown).

Silica-Gel TLC Analysis of Transported Steroids. Transported ^{3}H-labeled estrogens were analyzed by silica-gel TLC. The rates of flow for E_1, E_2, E_1S, 17β-estradiol 3-sulfate (E_2S), estrone 3-glucuronide, and 17β-estradiol glucuronide were 0.91, 0.84, 0.55, 0.48, 0.36, and 0.30, respectively. TLC of transported estrogens after 1- and 2-h incubations demonstrated a conversion of free estrogens into conjugated estrogens. Metabolites of [^{3}H]E_1 and [^{3}H]E_2 mainly consisted of E_1S and E_2S, respectively. There was no significant difference in the excretions of free [^{3}H]E_1 and [^{3}H]E_2 between the two cell lines (Fig. 5). In the transport assay of [^{3}H]E_1, excretion of E_1S was significantly greater in LLC/BCRP cells than that in LLC-PK1 cells (Fig. 5A). In the transport assay of [^{3}H]E_2, excretion of E_2S was significantly greater in LLC/BCRP cells than that in LLC-PK1 cells (Fig. 5B). In addition, there was no significant difference in the reabsorption of free

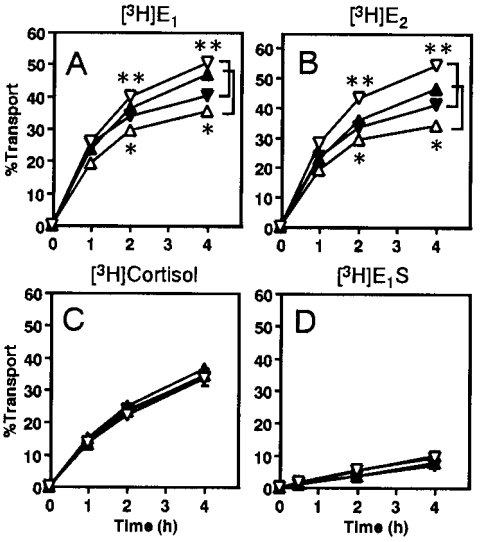

Fig. 3. Transcellular transport of ^{3}H-labeled steroids. Cells (2.4×10^6 /well) were plated on 3-μm pore filters 3 days before beginning the experiment. ^{3}H-labeled steroids were added in either the apical or the basal side of the medium. After 1, 2, and 4 h, the percentage of radioactivity that appeared in the opposite side was measured. A, transport of 10.6 nM [^{3}H]E_1. B, transport of 10.9 nM [^{3}H]E_2. C, transport of 15.6 nM [^{3}H] cortisol. D, transport of 25 nM [^{3}H]E_1S. △, excretion (basal-to-apical transport) in LLC-PK1; ▲, excretion in LLC/BCRP; ▽, reabsorption (apical-to-basal transport) in LLC-PK1; ▼, reabsorption in LLC/BCRP. The data, presented as a percentage fraction of the total radioactivity before incubation, are means ± S.D. of triplicate determinations from three different cultures. Where a vertical bar is not shown, standard deviation is within the symbol. *, $p < 0.05$ in the excretion; **, $p < 0.05$ in the reabsorption. The results were confirmed at least three times.

$[^3H]E_1$ and $[^3H]E_2$ between the two cell lines (data not shown).

The increased excretion of sulfated estrogens in LLC/BCRP cells was not caused by an activated conjugation reaction. Silica-gel TLC of the supernatant after incubation of the cell suspensions with $[^3H]E_1$ or $[^3H]E_2$ revealed no significant difference in activities that convert free estrogens into sulfate conjugates between LLC/BCRP and LLC-PK1 cells during a 2-h incubation (data not shown). Cell extracts were not used in this experiment because the radioactivity was less than 2% of that in the supernatant.

Collectively, exogenous BCRP expression did not affect transport of free $[^3H]E_1$ or $[^3H]E_2$, which was not what we originally expected. Exogenous BCRP expression in LLC/BCRP cells resulted in the increased excretion of E_1S or E_2S across the apical membrane of the cells. FTC efficiently suppressed the increased excretion of conjugated estrogens (E_1S or E_2S) in LLC/BCRP cells. These results suggest that E_1S and E_2S, but not free E_1 and E_2, are likely to be physiological substrates of BCRP.

Subcellular Localization of Exogenous BCRP in LLC-PK1 Cells. Immunohistochemical analysis of BCRP in LLC/BCRP cells showed very faint fluorescence in the membrane. Therefore, we constructed EGFP-BCRP to confirm the localization of BCRP in the transfected LLC-PK1 cells. Expression of an EGFP-BCRP fusion protein in LLC/EGFP-BCRP cells was detected as a 100-kDa band by Western blotting under reducing conditions with an anti-BCRP antibody (Fig. 6A). This 100-kDa protein also reacted with an anti-GFP antibody (data not shown). LLC/EGFP-BCRP cells displayed resistance to SN-38 and MXR and increased basal-to-apical transport of [^{3}H]MXR compared with LLC-PK1 cells (Fig. 6B). LLC/EGFP-BCRP cells showed increased basal-to-apical transport of ^{3}H-labeled estrogens compared with LLC-PK1 cells, although this transport activity was somewhat weaker than in LLC/BCRP cells (data not shown).

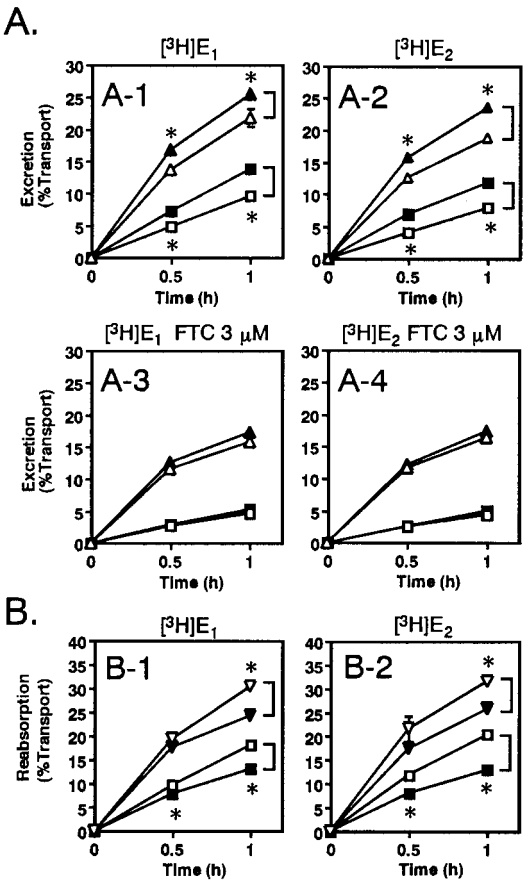

Fig. 4. Transcellular transport of ether-nonextractable metabolites of ^{3}H-labeled estrogen. The experimental procedure was the same as that of the transepithelial transport assay until sample collection. After 0.5 or 1 h of incubation, all of the medium in the opposite side was collected, and free estrogens were separated from estrogen metabolites by diethyl ether. Total radioactivity before the ether extraction and radioactivity in the aqueous fraction (estrogen metabolites) of transported ^{3}H-labeled estrogens are presented as percentage fractions of the radioactivity before incubation. A, excretion (basal-to-apical transport) of ^{3}H-labeled steroids. A-1, excretion of 10.6 nM $[^3H]E_1$ metabolites. A-2, excretion of 10.9 nM $[^3H]E_2$ metabolites. A-3, excretion of 10.6 nM $[^3H]E_1$ metabolites in the presence of 3 μM FTC. A-4, excretion of 10.9 nM $[^3H]E_2$ metabolites in the presence of 3 μM FTC. B, reabsorption (apical-to-basal transport) of ^{3}H-labeled steroids. B-1, reabsorption of 10.6 nM $[^3H]E_1$ metabolites. B-2, reabsorption of 10.9 nM $[^3H]E_2$ metabolites. △, total excretion in LLC-PK1; ▲, total excretion in LLC/BCRP; □, transport of water-soluble metabolites in LLC-PK1; ■, transport of water-soluble metabolites in LLC/BCRP; ▽, total reabsorption in LLC-PK1; ▼, total reabsorption in LLC/BCRP. The data, presented as percentage fractions of the total radioactivity, are means ± S.D. of triplicate determinations from three different cultures. Where a vertical bar is not shown, standard deviation is within the symbol. *, $p < 0.05$. The results were confirmed at least three times.

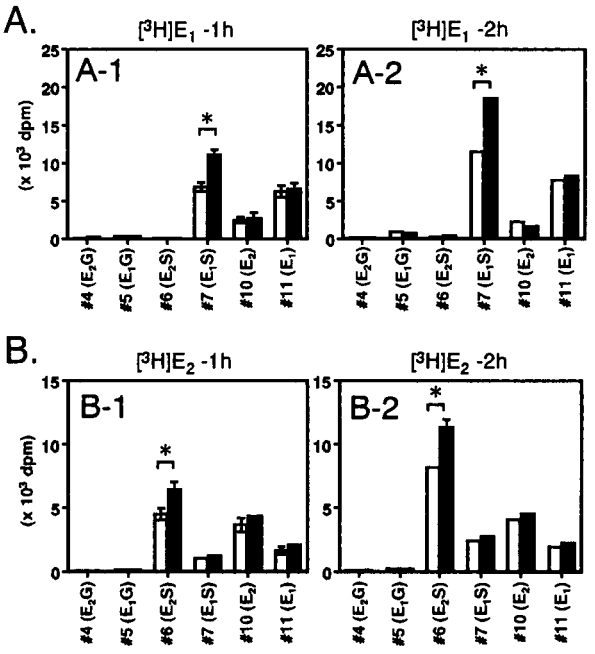

Fig. 5. Silica-gel TLC analysis of excreted ^{3}H-labeled estrogens and their metabolites. The experimental procedure was the same as that of the transepithelial transport assay until sample collection. Medium (50 μl) in the apical side after either a 1- or 2-h incubation was mixed with 100 μl of methanol, spotted, and run simultaneously with standard unlabeled estrogens and derivatives (100 μg each) on silica-gel plates in chloroform/methanol/acetic acid (8:3:1). Separated zones, visualized under ultraviolet, were cut, and their radioactivities were counted. A, excreted $[^3H]E_1$ and its metabolites after the addition of 10.6 nM $[^3H]E_1$. A-1, 1-h incubation. A-2, 2-h incubation. B, excreted $[^3H]E_2$ and its metabolites after the addition of 10.9 nM $[^3H]E_2$. B-1, 1-h incubation. B-2, 2-h incubation. □, LLC-PK1 cells; ■, LLC/BCRP cells; #4 to #11 indicate segments in the silica-gel TLC plate. The data are means ± S.D. of triplicate determinations from three different cultures. Where a vertical bar is not shown, standard deviation is within the symbol. *, $p < 0.05$; E_1G, estrone 3-glucuronide; E_2G, 17β-estradiol glucuronide. The results were confirmed twice.

EGFP-BCRP was identified in the apical membrane of the cells using fluorescence microscopy (Fig. 6C).

Intravesicular Transport Assay of ^{3}H-Labeled Compounds. Membrane vesicles (50 μg/reaction) from both K562 and K562/BCRP cells were incubated with [^{3}H]E_1 or [^{3}H]E_2 in the absence or presence of 5 mM ATP. No detectable uptake of [^{3}H]E_1 or [^{3}H]E_2 was observed regardless of the presence of ATP (Fig. 7A). In contrast, ATP-dependent uptake of [^{3}H]E_1S was observed in membrane vesicles (50 μg/reaction) from K562/BCRP, but not in those from K562. In the presence of 5 mM ATP, the amount of vesicle-associated [^{3}H]E_1S rapidly increased and reached a plateau level by 5 min. The maximum uptake of [^{3}H]E_1S at 50 nM was measured as approximately 10 pmol/min/mg protein. In the absence of ATP, no [^{3}H]E_1S uptake was detected. ATP-dependent uptake of [^{3}H]$E_2$17G was also detected in membrane vesicles from K562/BCRP and those from K562 (Fig. 7A). However, there was no difference in the uptake between K562/BCRP vesicles and K562 vesicles, suggesting that the ATP-dependent uptake of [^{3}H]$E_2$17G was mediated by transporters other than BCRP. SN-38 and FTC strongly inhibited the [^{3}H]E_1S uptake in membrane vesicles from K562/BCRP cells (Fig. 7B). These results also indicate that the ATP-dependent [^{3}H]E_1S uptake is BCRP-dependent.

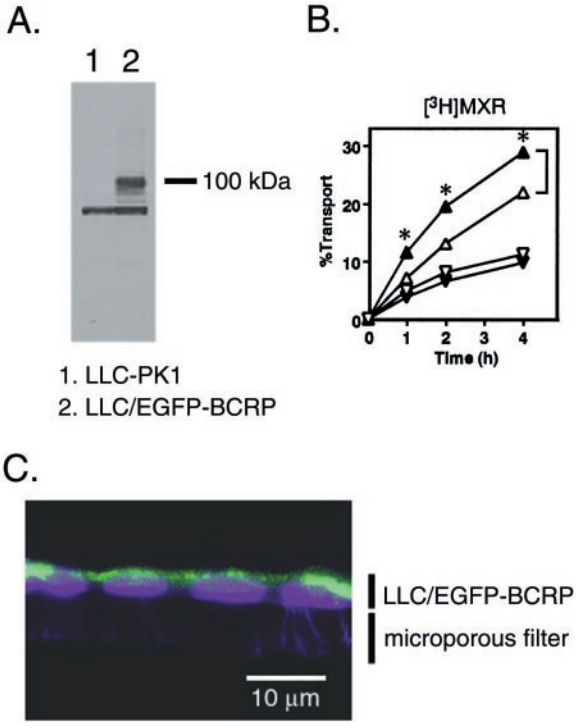

Fig. 6. Subcellular localization of exogenous BCRP in LLC/EGFP-BCRP cells. A, expression of EGFP-BCRP fusion protein in LLC/EGFP-BCRP cells. Western blotting with an anti-BCRP polyclonal antibody detected EGFP-BCRP as a 100-kDa band. B, transcellular transport of [^{3}H]MXR. The experimental procedure was the same as that used with the LLC/BCRP cells. △, excretion (basal-to-apical transport) in LLC-PK1; ▲, excretion in LLC/EGFP-BCRP; ▽, reabsorption (apical-to-basal transport) in LLC-PK1; ▼, reabsorption in LLC/EGFP-BCRP. The data, presented as a percentage fraction of the total radioactivity, are means of triplicate determinations from three different cultures. Error bars are within the symbols. *, $p < 0.05$ in the excretion. C, the sagittal view of the monolayer of LLC/EGFP-BCRP cells cultured on a 3-μm microporous filter membrane. EGFP-BCRP is detected by fluorescent microscopy as green fluorescence (λ_{ex}, 470–490 nm), and cell nuclei counterstained with 1.5 μg/ml 4′,6-diamidino-2-phenylindole (λ_{ex}, 330–385 nm) are indicated by purple fluorescence.

Characterization of ATP-Dependent [^{3}H]E_1S Uptake into K562/BCRP Membrane Vesicles. To confirm that the ATP-dependent association of [^{3}H]E_1S with K562/BCRP membrane vesicles represent transport into the intravesicular space, rather than the binding of [^{3}H]E_1S with the vesicle surface, the osmotic sensitivity of [^{3}H]E_1S uptake was examined. [^{3}H]E_1S uptake into K562/BCRP membrane vesicles significantly decreased when the sucrose concentration increased (Fig. 8A). The [^{3}H]E_1S uptake in the presence of 1 M sucrose was approximately 60% of that in the presence of 250 mM sucrose. This suggested that a portion of the [^{3}H]E_1S that precipitated with K562/BCRP membrane vesicles was in fact within these vesicles. In addition, the [^{3}H]E_1S uptake in K562/BCRP membrane vesicles was E_1S concentration-de-

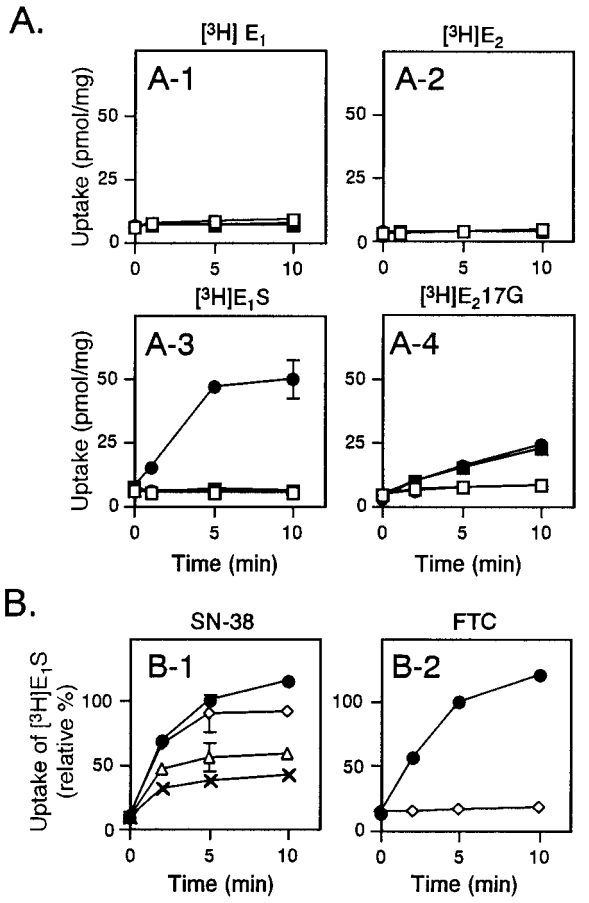

Fig. 7. Intravesicular uptake of estrogens and estrogen metabolites. A, ATP-dependent uptake. Membrane vesicles from K562 or K562/BCRP were incubated at 37°C with radiolabeled estrogens or estrogen metabolites in the absence or presence of 5 mM ATP. A-1, membrane vesicles (50 μg of protein) incubated with 31 nM [^{3}H]E_1. A-2, membrane vesicles (50 μg of protein) incubated with 27 nM [^{3}H]E_2. A-3, membrane vesicles (50 μg of protein) incubated with 50 nM [^{3}H]E_1S. A-4, membrane vesicles (200 μg of protein) incubated with 150 nM [^{3}H]$E_2$17G. Each point and vertical bar represents mean ± S.D. from triplicate determinations. □, uptake in K562 vesicles in the absence of ATP; ■, uptake in K562 vesicles in the presence of 5 mM ATP; ○, uptake in K562/BCRP vesicles in the absence of ATP; ●, uptake in K562/BCRP vesicles in the presence of 5 mM ATP. B, effect of BCRP substrate and inhibitor. K562/BCRP membrane vesicles (50 μg of protein) were incubated with 50 nM [^{3}H]E_1S in the absence (●) or presence of 3 μM (◇), 10 μM (△), or 20 μM (×) test compounds. B-1, SN-38. B-2, FTC. Data are represented as relative [^{3}H]E_1S uptake (percentage) of that in 5 min without inhibitors. Each point and vertical bar represents mean ± S.D. from triplicate determinations. Where a vertical bar is not shown, standard deviation is within the symbol.

pendent but saturable (Fig. 8B). The K_m and V_{max} values of this uptake were calculated by Eadie-Hofstee plots as 6.8 ± 1.4 μM and 1.4 ± 0.3 nmol/min/mg protein, respectively.

Effect of Physiological Steroids on E_1S Uptake. The effects of physiological steroids and their metabolites on BCRP-dependent [^{3}H]E_1S uptake were tested (Fig. 9). In a comparison of the two estrogens, E_2 showed a stronger inhibitory effect than did E_1. Among the estrogen derivatives examined, E_1S showed the strongest inhibitory effect. The inhibitory effects of E_2 and E_2S, however, proved to be almost the same. Estrogen glucuronides did not show any significant inhibition of BCRP-mediated [^{3}H]E_1S uptake, which was also the case for progesterone and cortisol. Dehydroepiandrosterone sulfate showed stronger BCRP inhibition than did dehydroepiandrosterone. Taurolithocholate and taurolithocholate sulfate showed stronger inhibition of the BCRP-mediated [^{3}H]E_1S uptake than did taurocholate. These results show that sulfate-conjugated steroids have high affinity with BCRP.

Discussion

LLC-PK1 cells have been used to characterize the drug-transporting properties of P-gp, MRP1, and BCRP because they retain high polarity in culture dishes. Exogenous P-gp and BCRP are expressed in the apical membrane, whereas exogenous MRP1 is expressed in the basolateral membrane of LLC-PK1 cells (Ueda et al., 1992; Evers et al., 1996; Jonker et al., 2000). In this study, we show that exogenous BCRP expression in LLC-PK1 cells resulted in increased [^{3}H]MXR transport in the basal-to-apical direction and that this was inhibited by either E_1, E_2, or FTC, but not by VRP. These results indicate that exogenous BCRP is localized in the apical membrane of LLC-PK1 cells. In addition, BCRP expression was visualized in the apical membrane of LLC/EGFP-BCRP cells as an EGFP-BCRP fusion protein. LLC/EGFP-BCRP cells also showed basal-to-apical transporting activity of [^{3}H]MXR and ^{3}H-labeled estrogens.

The transcellular transport assay of [^{3}H]E_1 and [^{3}H]E_2 showed BCRP-mediated increased excretion and decreased reabsorption of ^{3}H-labeled steroids. However, BCRP did not transport [^{3}H]E_1 and [^{3}H]E_2 in their primary forms. Silica-gel TLC demonstrated that [^{3}H]E_1 and [^{3}H]E_2 were rapidly converted into sulfate conjugates in both cell lines and that BCRP-dependent increased excretion and decreased reabsorption of ^{3}H-labeled estrogens was caused by BCRP-mediated transport of estrogen sulfates. The strong inhibition of increased excretion of ^{3}H-labeled estrogen metabolites by FTC in LLC/BCRP cells also indicated BCRP-mediated excretions of estrogen sulfates. When other steroids were analyzed, we found that *BCRP* transduction had no effect on the transport of cortisol and progesterone. Because cortisol is excreted by P-gp expressed in LLC-PK1 cells, P-gp activity was similar between LLC-PK1 and LLC/BCRP (Ueda et al., 1992). Therefore, the increased excretion of estrogen sulfates was not associated with P-gp in LLC/BCRP cells. Transcellular transport assays using [^{3}H]E_1S showed no difference in transport between LLC/BCRP and LLC-PK1. [^{3}H]E_1S cannot freely pass through cell membranes because it is highly hydrophilic. BCRP, expressed on the cell surface, mediates ATP-dependent transport of substrates from the intracellular space to the outside of the cells; therefore, it does not transport sulfated estrogens placed outside of the cells.

LLC-PK1 cells are epithelial cells of the proximal tubule of the porcine kidney and naturally express several transporters. In this study, parental LLC-PK1 cells excreted MXR beyond the reabsorption level, and this excretion was strongly suppressed by both FTC and estrogens. This excretion was barely inhibited by VRP, and these results are indicative of an endogenous MXR transporter in the apical membrane of LLC-PK1 cells. The results of the drug sensitivity test also suggested the existence of an endogenous transporter that effluxed MXR in LLC-PK1 cells. BCRP ex-

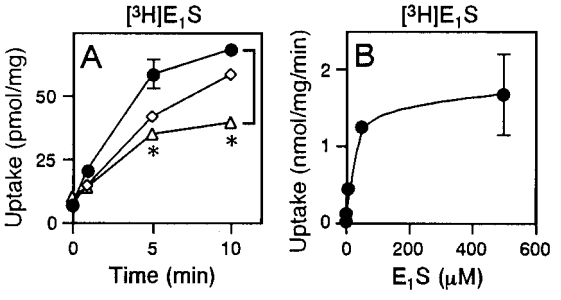

Fig. 8. Characterization of [^{3}H]E_1S uptake in K562/BCRP vesicles. A, osmotic sensitivity of [^{3}H]E_1S uptake. The K562/BCRP membrane vesicles (50 μg of protein) were incubated with 50 nM [^{3}H]E_1S in the presence of 250 mM (●), 500 mM (◇), or 1,000 mM (△) sucrose. B, concentration dependence of E_1S uptake. The K562/BCRP membrane vesicles (50 μg of protein) were incubated with 50 nM [^{3}H]E_1S in the absence or presence of unlabeled E_1S (0.5, 5, 50, or 500 μM). K_m and V_{max} values calculated from Eadie-Hofstee plots were 6.8 ± 1.4 μM and 1.4 ± 0.3 nmol/min/mg protein, respectively. Each point and vertical bar represents mean ± S.D. from triplicate determinations. Where a vertical bar is not shown, standard deviation is within the symbol. *, $p < 0.05$.

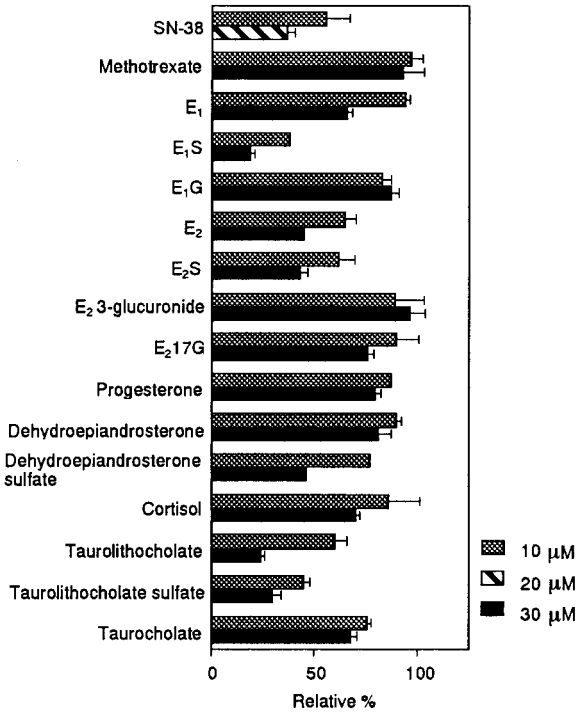

Fig. 9. Effect of physiological steroids on [^{3}H]E_1S uptake. Membrane vesicles (50 μg of protein) from K562/BCRP were incubated at 37°C with 50 nM [^{3}H]E_1S in the presence of the indicated concentration of steroid compounds. Data are represented as relative [^{3}H]E_1S uptake (percentage) of that of a 5-min control incubation. E_1G, estrone 3-glucuronide. The data are means ± S.D. of triplicate determinations. Where a horizontal bar is not shown, standard deviation is within the symbol.

pression analysis of mammals revealed that it was strongly expressed in the kidney, even more highly than in the placenta (Jonker et al., 2000). Taken together, we conclude from these data that an endogenous porcine homolog of BCRP was expressed in the apical membrane of LLC-PK1 cells. In addition, other transporters were expressed in the basolateral membrane of LLC-PK1 and LLC/BCRP cells and mediated the reabsorption of E_1S and E_2S. Reabsorption of ^3H-labeled estrogen metabolites increased in the presence of FTC and decreased in the presence of VRP (data not shown). Therefore, we also conclude that the transporters may contain a porcine homolog of MRP1 (Cole et al., 1994; Evers et al., 1996).

To further verify BCRP-mediated transport of estrogen and the corresponding metabolites, the uptake of ^3H-labeled estrogens in membrane vesicles from K562/BCRP cells was investigated. For this purpose, we chose a rapid centrifugation technique in which approximately 10% of the membrane protein was consistently recovered as a pellet in every reaction tube. The recovery rate of membrane protein was similar to that in a rapid filtration method using Millipore membranes (10–15%) (Naito et al., 1988). This figure seems to be low, but our recovery rate was always uniform, and the background radioactivity was usually approximately 1 to 2% of the total radioactivity in the reaction mixture. In our assay system, therefore, reproducible results with minor scattering were obtained.

BCRP-mediated uptake of [^{3}H]E_1 and [^{3}H]E_2 was not detected in membrane vesicle experiments (Fig. 7). ATP-dependent uptake of [^{3}H]E_1S was, however, observed in K562/BCRP membrane vesicles with a K_m value of 6.8 ± 1.4 μM but not in vesicles from K562 cells. This ATP-dependent uptake was inhibited by the BCRP substrate anticancer agent SN-38 and the BCRP inhibitor FTC, suggesting that the transport is mediated by BCRP (Fig. 7). When the sucrose concentration in the transport reaction mixture was increased to 1 M, the [^{3}H]E_1S uptake decreased to 60% of that measured in the presence of 250 mM sucrose. This result suggests that a fraction of [^{3}H]E_1S was actually incorporated into the inner space of K562/BCRP membrane vesicles. MRP1 is also known to transport E_1S stimulated in the presence of glutathione (Qian et al., 2001). The K_m values of E_1S transport by MRP1 in the presence or absence of 1 mM glutathione were reported to be 0.73 and 4.2 μM, respectively. In our system however, glutathione at 1 mM did not stimulate [^{3}H]E_1S uptake into K562/BCRP membrane vesicles (data not shown).

Various steroidal compounds were shown to inhibit [^{3}H]E_1S uptake into K562/BCRP membrane vesicles (Fig. 9). Among the noncharged steroids, E_2 showed a stronger inhibitory effect than either E_1, dehydroepiandrosterone, progesterone, or cortisol. Among steroid metabolites, glucuronides showed a marginal effect on [^{3}H]E_1S uptake into K562/BCRP membrane vesicles. The ATP-dependent uptake of [^{3}H]$E_2$17G did not seem to be mediated by BCRP (Fig. 7). Therefore, glucuronides are not likely to be substrates for BCRP. In contrast, E_1S, E_2S, dehydroepiandrosterone sulfate, taurolithocholate, and taurolithocholate sulfate strongly inhibited [^{3}H]E_1S uptake. These data suggest therefore that these sulfated steroidal compounds interact with BCRP and may be exported by BCRP.

Free E_1 and E_2 both blocked intravesicular E_1S uptake by BCRP (Fig. 9). Incubation of either [^{3}H]E_1 or [^{3}H]E_2 with K562/BCRP membrane vesicles for up to 10 min did not produce sulfated forms of [^{3}H]estrogens, as demonstrated by TLC (data not shown). These results suggest that free estrogens bind BCRP and may interfere with its interaction with sulfated estrogens, although free estrogens are not transported by BCRP, per se. We recently reported the blocking of BCRP-mediated anticancer drug transport by synthetic estrogen agonists and antagonists (Sugimoto et al., 2003). They do not have steroid structures, and some of them do not have residues to be sulfated. Therefore, at least some of these compounds block the function of BCRP in these native forms. It is not clear, however, whether these antiestrogens would be transported by BCRP.

We hitherto have provided evidence that indicates BCRP-mediated export of estrogen sulfates. Very recently, other groups have reported intravesicular uptake of estrogen metabolites by membrane vesicles from BCRP-expressing cells (Chen et al., 2003; Suzuki et al., 2003). BCRP is widely expressed in tissues and organs such as intestine, liver, hematopoietic stem cells, and vessels other than urogenital organs, and the physiological function of BCRP is not likely to be restricted to the transport of estrogen sulfates (Doyle et al., 1998; Maliepaard et al., 2001; Zhou et al., 2001). To our knowledge, E_1S is the first compound identified as a physiological substrate transported by BCRP with a similar affinity and capacity as MRP1. Recently, it was reported that *Bcrp1*−/− mice did not manifest any detectable gross abnormalities, were fertile, and produced litters of normal size (Zhou et al., 2002). However, another lineage of *Bcrp1*−/− mice displayed photosensitivity, and indeed, BCRP has been implicated in the transport of chlorophyll-derived dietary phototoxin and protoporphyrin in hematopoietic stem cells and erythrocytes (Jonker et al., 2002). Further investigation will be needed to thoroughly clarify the physiological role of BCRP.

Cells expressing point mutants of BCRP containing Thr482, Gly482, or Met482 instead of Arg482 (wild-type) have been found to show altered substrate recognition and increased drug resistance against MXR and doxorubicin (Honjo et al., 2001; Ozvegy et al., 2002; Wang et al., 2003). We therefore started to examine the functional consequences of BCRP Arg482 mutations and recently made 15 Arg482 mutant *BCRP* cDNA transfectants. Surprisingly, 13 of 15 mutants showed higher resistance to MXR and doxorubicin (M. Miwa, S. Tsukahara, E. Ishikawa, S. Asada, Y. Imai, and Y. Sugimoto, unpublished findings). The transporting activity of estrogen sulfates by these mutant BCRPs is now under further investigation in our laboratory. In conclusion, we find that BCRP is not associated with the transport of free E_1 and E_2, but it can physiologically transport sulfated conjugates of estrogens.

Acknowledgments

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