Contribution of Ion-Pair Complexation with Bile Salts to the Transport of Organic Cations across LLC-PK1 Cell Monolayers

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Purpose. To examine the effect of ion–pair complexation with endogenous bile salts on the transport of organic cations (OCs) across LLC-PK1 cell monolayers.

Methods. The transport of tributylmethyl-ammonium (TBuMA) and triethylmethylammonium (TEMA) across the cell monolayer was measured in the presence of taurodeoxycholate (TDC), an endogenous organic anion that forms an ion-pair complex with TBuMA, but not with TEMA.

Results. Under proton gradient conditions (i.e., pH 6.0 apical/pH 7.4 basal), the above OCs exhibited similar transport charactersistics, consistent with the well-established OC/H^+ antiporter, and the presence of TDC had no measurable effect on the transport of these OCs. Under pH-equilibrated conditions (i.e., pH 7.4 apical/pH 7.4 basal); however, basal to apical transport of TBuMA, not that of TEMA, was increased in the presence of TDC, probably as a result of the formation of a lipophilic ion-pair complex between TBuMA and TDC. The transport and efflux of the TBuMA-TDC complex across the apical membrane of the cell was inhibited by representative substrates of the P-glycoprotein (P-gp), indicating the involvement of P-gp in this process. The increased affinity of the ion-pair complex to P-gp is consistent with a mechanism involving increased transport.

Conclusion. In cases where there is no proton gradient between the plasma and urine, the formation of lipophilic ion-pair complexes in the kidney with endogenous bile salts might be involved in the *in vivo* urinary excretion of large Mw OCs, such as TBuMA.

KEY WORDS: ion-pair complex; P-glycoprotein; LLC-PK1 cells; TbuMA; taurodeoxycholate (TDC).

INTRODUCTION

Many drugs and endogenous metabolites contain primary, secondary, and tertiary amines or quaternary ammonium structures that are positively charged at physiologic pH and exist as organic cations (OCs). Examples of such OCs include drugs from various clinical classes, such as antihistamines, skeletal muscle relaxants, antiarrhythmics, and β-adrenoreceptor blocking agents, and a number of endogenous bioactive compounds, such as dopamine, choline, and *N*-methylnicotinamide. Because of the positive charge on the molecules, membrane transporters, such as the OC/H^+ antiporter and P-glycoprotein (P-gp), are generally involved in their absorption, distribution, and elimination (1).

A molecular weight (Mw) threshold (i.e., 200 ± 50, in rats, for example) has been suggested for the appreciable (more than 10%) biliary excretion of OCs (2). Consistent with this proposal, tributylmethyl ammonium (TBuMA, Mw 200) is significantly excreted (35% of an iv dose of 12 μmol/kg) into the bile in rats, whereas the excretion of triethylmethyl ammonium (TEMA, Mw 116), a structural analogue of TBuMA, is negligible (0.17% of the same dose) (3). The Mw threshold can be attributed to the fact that TBuMA forms lipophilic ion-pair complexes with endogenous bile salts, whereas TEMA does not (4). Taurodeoxycholate (TDC) has been proposed as one of bile salts that most efficiently form lipophilic ion-pair complexes with large Mw OCs like TBuMA. As a result of the formation of an ion-pair complex, TBuMA-TDC, the apparent partition of TBuMA to *n*-octanol from phosphate buffer (pH 7.4) was dramatically increased (from practically no partition to nearly complete partition; Refs. 4,5). Owing to that increase in the lipophilicity, the affinity of the ion-pair complex to P-gp, as well as passive diffusion, was increased, subsequently leading to a substantial biliary excretion of TBuMA (4). Thus, the formation of ion-pair complexes has been proposed as a mechanism for the preferential biliary excretion of high Mw OCs (4,6).

Bile salts exist in the blood and urine at considerably high concentrations in obstructive cholestasis in SD rats (about 402 and 328 μM for the blood and urine, respectively; Ref. 7) and in women of intrahepatic cholestasis (up to 486 μM for the urine; Ref. 8), implying the significant presence of bile salts in the kidney, especially in patients with hepatic dysfunction. Thus, it can be hypothesized that large Mw OCs may form lipophilic ion-pair complexes with specific bile salts in the kidney. This hypothesis is supported by our preliminary data showing that the apparent partition coefficient of TBuMA, not that of TEMA, between pH 7.4 phosphate buffer and *n*-octanol, is significantly increased in the presence of the organic anion (OA) fraction of kidney extracts (i.e., from 0.1 to 4.3 for 1 μM of the OCs, n = 3). The formation of ion-pair complexes, therefore, might influence the renal excretion of OCs in the same manner as it influences the biliary excretion of OCs (4). In the present study, therefore, we report on the study of the effect of ion-pair complexation on the renal excretion of OCs. For this purpose, the vectorial transport of OCs across LLC-PK1 cell monolayers was examined in the presence of TDC. The LLC-PK1 cell monolayer, which expresses functional activities of the OC/H^+ antiport (9,10) and the P-gp system (11,12), but not the bile salt export pump (Bsep) (13), was selected as a model membrane for renal proximal tubules (9).

MATERIALS AND METHODS

Materials

[^{3}H]TBuMA (0.5 Ci/mmol) and [$_3$H]TEMA (0.5 Ci/mmol) were synthesized as previously described (4). [^{3}H]Daunomycin (4.4 Ci/mmol), [^{14}C]TEA (tetraethylammonium, 24 mCi/mmol), [^{3}H]taurocholate (2.0 Ci/mmol), and [^{14}C]mannitol (50 mCi/mmol) were purchased from NEN Life Science Products (Boston, MA, USA). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Trypsin–EDTA was purchased from Gibco Laboratories (Gaithersburg, MD, USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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Cell Culture

A porcine kidney epithelial cell line, LLC-PK1 cells, was purchased from the American Type Culture Collection (Rockville, MD, USA). LLC-PK1 cells from 210 to 225 serial passages were cultured in Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum, 5 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. For the transport experiments, cells were grown on a permeable polycarbonate insert (1cm^2, 0.4-μm pore size, Corning Costar Co., Cambridge, MA, USA) in 12-Transwell plates (4 cm^2, Corning Costar Co.) at a density of 5×10^5 cells/cm^2, and the medium was changed at 2-day intervals. For the cellular uptake or efflux experiments, cells were grown on 12-Transwell plates at a density of 5×10^5 cells/cm^2, and the medium was changed at 2-day intervals.

Characterization of LLC-PK1 Cell Monolayers

To confirm the functional activities of the LLC-PK1 cells, the vectorial transport of TEA, daunomycin, taurocholate, and mannitol was measured using LLC-PK1 cell monolayers grown in Transwell inserts 3, 5, and 7 days after seeding. The transport medium consisted of Hank's balanced salt solution (HBSS) buffer supplemented with 20 mM of glucose, 9 mM of sodium bicarbonate, and 25 mM of HBSS (pH 7.4). The transport medium with a pH of 6.0 was prepared by replacing *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfonic acid with equimolar 2-*N*-moroholine-ethansulfonic acid in the HBSS buffer. The transepithelial electrical resistance (TEER) value was determined using an EVOM™ epithelial volt/ohm-meter (World Precision Instruments, Sarasotas, FL, USA) to evaluate the integrity of the monolayers. For measurement of the apical to basal (A-B) transport of TEA, 0.5 mL of the transport medium (pH 6.0) containing [^{14}C]TEA (10 μM, 0.12 μCi) was added on the apical side and 1.5 mL of the transport medium (pH 7.4) without TEA was added on the basal side of the insert. The insert was transferred to a well containing fresh transport medium every 30 min for 2 h. The radioactivity of an aliquot (0.3 mL) of the basal side of each well was determined by liquid scintillation counter (Wallac 1409, Perkin–Elmer Life Science Inc., Boston, MA, USA). For measurement of the basal to apical (B-A) transport, 1.5 mL of the transport medium (pH 7.4) containing [^{14}C]TEA (10 μM, 0.36 μCi) was added on the basal side, and 0.5 mL of the transport medium (pH 6.0) without TEA was added on the apical side. The transport medium in the apical side was replaced with 0.3 mL of fresh incubation medium every 30 min for 2 h. The radioactivity of an aliquot (0.3 mL) of the apical side after each replacement was measured. For the measurement of vectorial transport of daunomycin, taurocholate and mannitol, the experimental conditions were identical except the substrate concentration, i.e., 0.5 μM (1.1 μCi) [^{3}H]daunomycin, 1 μM (1.0 μCi) [^{3}H]taurocholate, and 10 μM (0.75 μCi) [^{14}C]mannitol, and the pH of the transport medium in apical side (pH 7.4).

Vectorial Transport of Organic Cations in the Presence of Bile Salts

For measurement of A-B transport, 0.5 mL of the transport medium containing [^{3}H]TBuMA or TEMA (10 μM, 0.5 μCi each) in the presence or absence of 100 μM TDC was added on the apical side (pH 7.4 for a pH equilibrated condition and pH 6.0 to make a proton gradient) and 1.5 mL of the transport medium without TBuMA or TEMA was added on the basal side of the insert. For measurement of the B-A transport, 1.5 mL of the transport medium containing [^{3}H]TBuMA or TEMA (10 μM, 1.5 μCi each) in the presence or absence of 100 μM TDC was added on the basal side (pH 7.4). To examine the concentration dependency of the B-A transport of TBuMA or TEMA, 1.5 mL of the transport medium (pH 7.4) containing [^{3}H]TBuMA or TEMA of given concentration (1–1000 μM) was added on the basal side of the insert and 0.5 mL of the transport medium without TBuMA or TEMA was added on the apical side (pH 7.4 for a pH equilibrated condition and pH 6.0 to make a proton gradient). To characterize the B-A transport of TBuMA-TDC complex, 1.5 mL of the transport medium containing [^{3}H]TBuMA of given concentration (1–1000 μM) in the presence of 10-fold molar excess of TDC was added on the basal side. To evaluated cell toxicity of the ingredient like TDC, 10 μM [^{14}C]mannitol was added together in all cases and after the experiments TEER value was checked. The radioactivity of the aliquot was counted by dual counting mode. The subsequent procedures were identical to the measurement of TEA transport above mentioned.

Vectorial Transport of Organic Cations in the Presence of Various Compounds

To elucidate the mechanism of the increased transport of TBuMA in the presence of TDC, the inhibitory effect of various compounds on the transport of TBuMA was measured by addition of 100 μM of various compounds such as daunomycin, verapamil, cyclosporin A, TEA, taurocholate and penicillin G to the transport medium, which contained 10 μM [^{3}H]TBuMA (0.5 μCi) and 100 μM TDC. The potential involvement of P-gp in the increased uptake of TBMA-TDC complex was examined by measuring the inhibitory effect of various concentrations of TBuMA-TDC complex (prepared by using 1, 10, and 100 μM TBuMA and a 10-fold molar excess of TDC in the transport medium) on the transepithelial transport of 0.5 μM (1.1 μCi) [^{3}H]daunomycin.

Uptake or Efflux of Organic Cations across the Apical Membrane

For measurement of the cellular uptake of TBuMA in the presence or absence of TDC from the apical side of the cells, cells in 12-Transwell plate were incubated with 2 mL of transport medium containing 10, 100 μM [^{3}H]TbuMA, and a 10-fold molar excess of TDC for 30 min at 37°C. The cells were washed twice with 2 mL ice-cold transport medium. After aspiration of the transport medium, the cells were digested by adding 1 mL of cell digestive solution (0.1% w/v Triton-X 100 in 0.3 N NaOH). The radioactivity of a 300-μL aliquot of the digested cell solution was measured. To examine the effect of various compounds on the efflux of TBuMA-TDC complex from the apical side of the cells, cells in 12-Transwell plate were preincubated with 2 mL of transport medium containing 100 μM [^{3}H]TBuMA and 1 mM TDC for 30 min at 37°C. The cells were washed rapidly twice with 2 mL of ice-cold transport medium and 2 mL of transport me-

dium containing 100 μM of various compounds, such as daunomycin, verapamil, TEA, taurocholate, and penicillin G, was added. The incubation medium in the 12-Transwell plate was replaced with 1.6 mL of fresh transport medium containing various compounds every 30 s for 2 min. The radioactivity of a 300-μL aliquot of the incubation medium was measured.

Calculations

In each transport experiment, the total amount of substrates transported across the cell monolayers was plotted against time and the mean transport rate (pmol/h/cm^2) was calculated from the linear portion of the curve (slope of the plot). The mean transport rate was then plotted against the initial concentration of the substrate in the transport medium, and the resulting profile was fitted to equation 1 to estimate the parameters for the mixed transport process involving saturable and linear kinetics. A weighted nonlinear regression analysis was performed in the fitting using WinNonlin (version 3.1; Pharsight Co., Mountainview, CA, USA).

$$V = V_{max} \times S/(K_m + S) + CL_{linear} \times S \quad (1)$$

Where V is the apparent linear initial rate (pmol/h/cm^2), S the initial concentration (μM) in the donor compartment of the insert, V_{max} and K_m are the maximum transport rate and the Michaelis–Menten constant, respectively, and CL_{linear} represents the linear clearance. The intrinsic clearance for the transport (CL_{int}) was obtained from V_{max}/K_m. All data are expressed as the means ± SD. The statistical significance of differences between treatments was evaluated using the unpaired Student's t test, and a value of $p < 0.01$ was considered to be statistically significant.

RESULTS

Characteristics of LLC-PK1 Cell Monolayers

The transport activity of LLC-PK1 cell monolayers was confirmed by measurement of the transepithelial transport of TEA, daunomycin, taurocholate and mannitol, which are the representative substrates for OC/H$^+$ antiporter (9,10), P-gp (14), and Bsep (14) and a well-known indicator of membrane leakage. As shown in Fig. 1, on day 3 after the seeding of the cell, the B-A transport of daunomycin was relatively low, but a marked increase in the transport was observed on day 5, consistent with the confluency of the cell monolayer. On day 7, however, a significant increase was observed for the transport of mannitol. Based on the above findings, all subsequent transport studies were carried out with the LLC-PK1 cell monolayers 5 days after cell seeding. The TEER value of the LLC-PK1 cell monolayers reached 170–210 Ω·cm^2 on day 5.

The B-A transport of 10 μM [^{14}C]TEA was 5.9-fold higher than that of the opposite direction in the presence of a proton gradient, consistent with proton gradient dependent transport of OCs via the OC/H$^+$ antiporter in LLC-PK1 cell monolayers (9,10). The B-A transport of 0.5 μM [^{3}H]daunomycin, a representative substrate of P-gp, was 6.1-fold greater compared to the A-B transport (on day 5), consistent with the operation of the P-gp system in the LLC-PK1 cells (11,12). This is supported by our previous study, in which the B-A trasnsport of rhodamine 123, another typical substrate for P-gp, across the LLC-PK1 cell (i.e., K_m: 16.2 ± 4.1 μM and

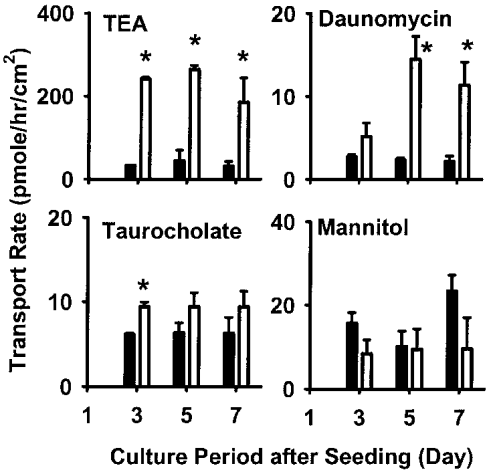

Fig. 1. Effect of culture period after the seeding of cells on the transepithelial transport of TEA in the presence of a proton gradient (pH 6.0 apical/pH 7.4 basal), daunomycin, taurocholate, and mannitol under pH- equilibrated conditions (pH 7.4 apical/pH 7.4 basal) across LLC-PK1 cell monolayers. Closed bars indicate the apical to basal transport, and open bars the basal to apical transport. Data represent the mean± SD of three determinations. *$p < 0.01$ compared with the A-B transport by unpaired Student t test.

V_{max}: 608 ± 100 pmol/h/cm^2) was similar to its transport across a P-gp developed cell line (15) (i.e., K_m: 12.8 ± 3.7 μM and V_{max}: 571 ± 100 pmol/h/cm^2 for Caco-2 cells). The presence of Bsep, which is responsible for the active excretion of bile acids in hepatocytes, appears to be unlikely in LLC-PK1 cells 5 days after the seeding, consistent with a previous report (13), because no significant difference between the A-B and B-A transports of taurocholate was observed for the cell (i.e., $p > 0.01$, Fig. 1C). The transport of 10 μM [^{14}C]mannitol in 1 h was less than 1.0% of the, corresponding to an apparent permeability (P_{app}) value of 7.0–14 × 10^{-7} cm/s under the given culture conditions, consistent with a previous report (9). In summary, the LLC-PK1 cell monolayer system developed here seemed to be appropriate for examining the transport of substrates via the OC/H$^+$ antiporter and the P-gp systems.

Effect of Bile Salts on the Vectorial Transport of Organic Cations

The vectorial transport of [^{3}H]TBuMA or [^{3}H]TEMA (10 μM each) across LLC-PK1 cell monolayers were measured as a function of time in the presence or absence of 100 μM TDC. We have previously reported that TBuMA is a substrate for both P-gp and OC/H$^+$ antiporter, whereas TEMA is a substrate only for OC/H$^+$ antiporter (6). Consistent with the fact that TBuMA and TEMA are substrates of OC/H$^+$ antiporter, the B-A transport of both OCs (10 μM each), in the presence of a proton gradient (i.e., pH 6.0 apical/pH 7.4 basal), was greater (i.e., 2.8 and 3.8-fold, respectively, in transport rates) than the corresponding A-B transport (Fig. 2A). The B-A transport of both OCs might be decreased when measured in the absence of a proton gradient (i.e., pH 7.4 apical/pH 7.4 basal). As the result, the difference between the transports of TEMA in the two directions disappeared (Fig. 2B for TEMA). However, the difference between the transport of TBuMA in the two directions still remained (Fig. 2B for TBuMA), consistent with the fact that TBuMA is also

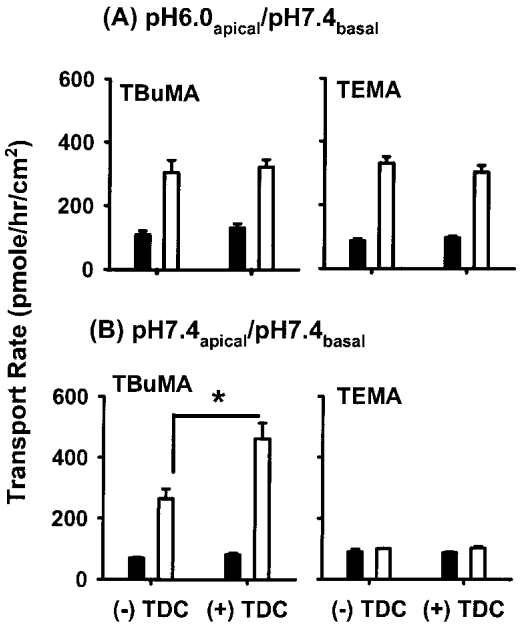

Fig. 2. Effect of TDC (100 μM) on the transepithelial transport of TBuMA (10 μM) and TEMA (10 μM) across the LLC-PK1 cell monolayers in the presence (A, pH 6.0 apical/pH 7.4 basal) and absence (B, pH 7.4 apical/pH 7.4 basal) of a proton gradient. Closed bars indicate the apical to basal transport, and open bars the basal to apical transport. Data represent the mean± SD of three determinations. *$p < 0.01$.

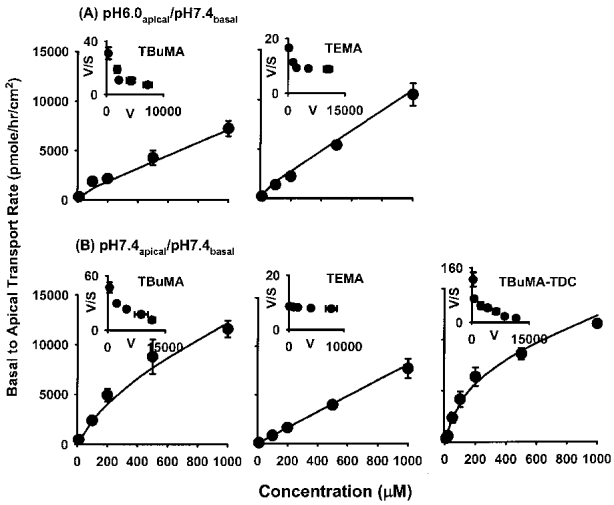

Fig. 3. Concentration-dependent B-A transport of TBuMA (1–1000 μM), and TEMA (1–1000 μM) and TBuMA-TDC complex (1–1000 μM TBuMA and 10-fold molar excess of TDC) across the LLC-PK1 cell monolayers in the presence (A, pH 6.0 apical/pH 7.4 basal) and absence (B, pH 7.4 apical/pH 7.4 basal) of a proton gradient. Data represent the mean± SD of three determinations. Insets represent Eadie–Hofstee transformations of corresponding data. Where V and S represent the transport rate and the concentration of OCs, respectively.

a substrate for P-gp. In the presence of a proton gradient, the presence of 100 μM TDC in the apical or basal side had no influence on the A-B or B-A transport of TBuMA and TEMA (Fig. 2A). In the absence of a proton gradient, the presence of 100 μM TDC had no effect on the A-B and B-A transport of TEMA (Fig. 2B), consistent with the absence of chemical interactions between TEMA and TDC (4). However, the B-A transport, but not the A-B transport, of TBuMA was increased by 1.8-fold in the presence of 100 μM TDC in the donor compartment, thereby maintaining a 3.3-fold difference in transport rate in the two directions (Fig. 2B). The TBuMA and B-A direction-specific effect of TDC appears to be associated with TBuMA-specific ion-pair formation of TDC (4) and an increased transport of the ion-pair complex via relevant transporter(s). The presence of 100 μM TDC appeared to have no influence on the integrity of the cell membranes, because no changes in the leakage of mannitol and TEER values were observed (data not shown).

Concentration Dependency of the Basal to Apical Transport of Organic Cations

Because the involvement of transport systems was observed for the B-A transport of TBuMA and TEMA, the nature and characteristics of the transport was further investigated. In the presence of a proton gradient, a concentration dependency was found for the transport of TBuMA and TEMA (Fig. 3A). An Eadie-Hofstee transformation (inset of Fig. 3) of the data shows that the B-A transport is mediated both by saturable and linear processes, and a kinetic analysis based on equation 1 yielded apparent K_m, V_{max}, CL_{int}, and CL_{linear} values for the saturable transport and linear process, respectively (Table I). The apparent V_{max} values for TBuMA and TEMA were comparable, whereas the apparent K_m value was 2.3-fold larger for TBuMA compared to TEMA. As a result, the CL_{int} (i.e., *in vitro* intrinsic clearance calculated from V_{max}/K_m) of TEMA was 2.7-fold larger than that of TBuMA. In addition, a 1.9-fold larger CL_{linear} was obtained for TEMA compared to TBuMA, possibly associated with larger diffusivity of the smaller compound, TEMA, in the cell membrane. These results indicate that TEMA is more efficiently transported than TBuMA across LLC-PK1 cell monolayers, probably via the OC/H^+ antiport system and passive mechanisms, in the presence of a proton gradient.

Under pH equilibrated conditions, the transport rate of TEMA increased as its concentration increased (Fig. 3B), indicating that carrier mediated system(s) were not involved in the transport. The CL_{linear} value for the linear transport (i.e., 8.1 μL/h/cm^2) was comparable to that (i.e., 10.2 μL/h/cm^2) in the presence of a proton gradient (Table I). The B-A transport of TBuMA under pH equilibrated conditions, on the other hand, exhibited a saturation as its concentration in the transport medium increased (Fig. 3B). This is distinct from the case of TEMA, indicating the involvement of transport system(s) other than OC/H^+ antiporter in the transport of TBuMA. The B-A transport of TBuMA under pH equilibrated conditions was more efficient (Fig. 2B) compared to that in the presence of a proton gradient (Fig. 2A), as indicated by the larger CL_{int} value in this condition (Table I).

Concentration Dependency of the Basal to Apical Transport of Ion-Pair Complexes

Under pH equilibrated conditions, the B-A transport of TBuMA was increased in the presence of TDC (Fig. 2B). This transport was further investigated in terms of the effect of the concentration of TBuMA-TDC ion-pair complex on the B-A transport rate of TBuMA. The concentration of ion-pair com-

Table I. Apparent Kinetic Parameters for the Basal to Apical Transport of Organic Cations in LLC-PK1 Cell Monolayers[a]

	TBuMA	TEMA	TBuMA-TDC
Proton gradient condition[b]			
K_m (μM)	45.9 ± 3.4	19.7 ± 4.1[d]	ND
V_{max} (pmole/hr/cm^2)	814 ± 54	918 ± 66	ND
CL_{int} (μl/hr/cm^2)	17.8 ± 1.4	48.5 ± 10[d]	ND
CL_{linear} (μl/hr/cm^2)	5.49 ± 0.34	10.2 ± 3.2[d]	ND
pH-equilibrated condition[c]			
K_m (μM)	349 ± 103	—	160 ± 39[e]
V_{max} (pmole/hr/cm^2)	8620 ± 1300	—	9980 ± 1600
CL_{int} (μl/hr/cm^2)	26.5 ± 9.7	—	64.7 ± 15[e]
CL_{linear} (μl/hr/cm^2)	6.86 ± 4.2	8.11 ± 0.15	10.2 ± 2.1[e]

ND, not determined.
[a] All data represent the mean ± SD (n = 3).
[b] pH 6.0 apical/pH 7.4 basal.
[c] pH 7.4 apical/pH 7.4 basal.
[d] Statistically different from the case for TEMA ($p < 0.01$).
[e] Statistically different from the case for TBuMA ($p < 0.01$).

plexes is determined by the concentrations of the relevant counterions (i.e., OCs and OAs). In the case of TBuMA and TDC, the concentration of the TBuMA-TDC complex can be assessed by measurement of the concentration of TBuMA, as long as TDC is present in excess compared to TBuMA (4,5). For example, in the presence of a 10-fold excess of TDC (in molar concentration), more than 75% of the total TBuMA in the transport medium exists as the TBuMA-TDC ion-pair complex (4,5). In this case, the total concentration of TBuMA would represent the concentration of the ion-pair complex. In the present study, the concentration of TBuMA in the transport medium was varied over the range of 1–1000 μM, and the concentration of TDC in each medium was adjusted to so as to be 10-fold higher compared with TBuMA. Under the given conditions, the transport of TBuMA in the B-A direction exhibited a curvilinear increase as the concentration of TBuMA (and subsequently the concentration of TDC) increased (Fig. 3B for TBuMA-TDC). A kinetic analysis based on equation 1 revealed that the V_{max} value was unchanged, whereas the K_m value was significantly decreased (54%) by the presence of 10-fold TDC, resulting in a 2.4-fold increase in the CL_{int} for the transport of TBuMA. The decrease in the K_m value indicates an increase in the affinity of TBuMA to the relevant transporter, consistent with the formation of a lipophilic ion-pair complex with TDC (4). The CL_{linear} value of TBuMA was increased by 1.5-fold in the presence of excess TDC. The increase in the lipophilicity of TBuMA, through forming an ion-pair complex with TDC, might have contributed to the increase in the passive diffusion of TBuMA via the transcellular pathway. In this experiment, a significant decrease in TEER values (11 and 23% decrease), without influencing the mannitol leakage (data not shown), was observed by the presence of excess TDC (i.e., 5 and 10 mM, respectively) after the experiments. Despite the decrease in TEER values, however, paracellular diffusion does not appears to be involved in the increase of the CL_{linear} value of uncomplexed TBuMA, since the permeability of mannitol, a compound with a similar molecular weight and lipophilicity with TBuMA, was not changed. Above results indicate that a carrier mediated process, as well as passive diffusion, is increased in the presence of excess TDC.

Mechanisms of the Transport of Ion-Pair Complexes

To elucidate the mechanism responsible for the accelerated B-A transport of TBuMA in the presence of TDC, the involvement of various transport systems in the acceleration was examined under pH-equilibrated conditions. For this purpose, the effect of daunomycin, verapamil, and cyclosporin A (all P-gp substrates), TEA (an OC), taurocholate (a bile salt), and penicillin G (an OA) on the vectorial transport of TBuMA (10 μM) in the presence of TDC (100 μM) was examined. In this experiment, the concentration of each substrate in the transport medium was adjusted to 100 μM. In the absence of substrates (i.e., control), a several fold larger transport rate was observed for the B-A direction compared to A-B direction, as described above. The addition of 100 μM TEA, taurocholate and penicillin G had no effect on the transport of TBuMA in either direction, indicating that the accelerated transport of TBuMA is not associated with relevant transporters (i.e., OC transporter, Bsep or OA transporter, for example). On the other hand, 100 μM daunomycin, verapamil and cyclosporine A inhibited B-A transport and increased A-B transport (Fig. 4A), suggesting an involvement of P-gp in the accelerated transport of TBuMA.

The involvement of P-gp was further examined by measuring the effect of the TBuMA-TDC complex on the transport of 0.5 μM [^{3}H]daunomycin. The B-A transport of daunomycin was 5.1-fold larger than the A-B transport, consistent with the involvement of P-gp in the B-A transport of daunomycin (Fig. 4B). The B-A transport of daunomycin was inhibited in the presence of 10 μM TBuMA, consistent with the fact that TBuMA is also a substrate for P-gp (6). The presence of 100 μM TDC had no effect on the transport of daunomycin (Fig. 4B), indicating the absence of an interaction between the transport of daunomycin and Bsep. The fact that the B-A transport rate of 1 μM [^{3}H]taurocholate was not influenced by the presence of TBuMA-TDC (10: 100 μM) complex (data not shown) confirms the above conclusion. On the contrary, the B-A transport of daunomycin decreased significantly, with the simultaneous increase in A-B transport, as the TBuMA-TDC concentration in the medium increased (Fig. 4B). This suggests that daunomycin and TBuMA-TDC

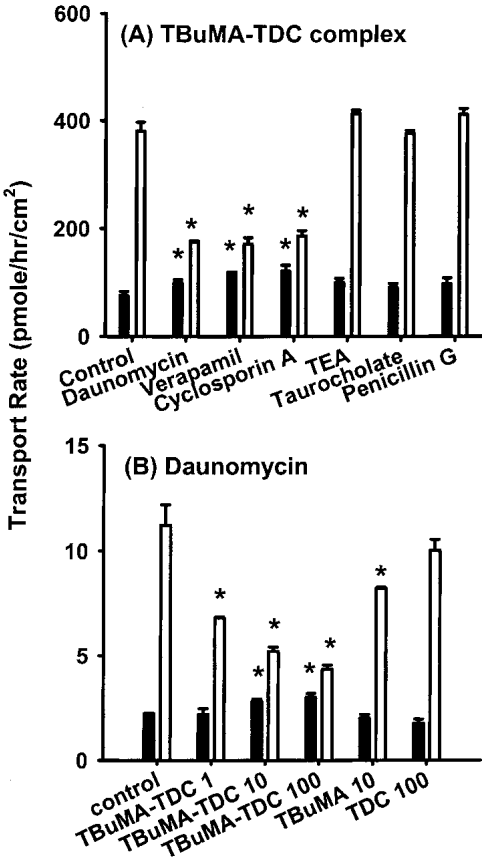

Fig. 4. (A) Effect of 100 μM daunomycin, verapamil, cyclosporin A, TEA, taurocholate, and penicillin G on the B-A transport rate of 10 μM TBuMA in the presence of 100 μM TDC under pH-equilibrated conditions. (B) Effect of various concentrations of TBuMA-TDC complex (produced by 1, 10, and 100 μM TBuMA and a 10-fold molar excess of TDC) on the B-A transport rate of 0.5 μM daunomycin under pH-equilibrated conditions. Closed bars indicate A-B transport, and open bars indicate B-A transport. Data represent the mean± SD of three determinations. *$p < 0.01$ compared with respective control values by unpaired Student *t* test.

might share a common transporter, probably P-gp, in their B-A transport.

Mechanisms of the Cellular Uptake and Efflux of Ion-Pair Complexes

P-gp is generally known to be expressed on the apical side of cell membranes (1,14), operating as a barrier for the apical uptake of its substrates through pumping them out. In this regard, more information on the role of P-gp could be obtained from experiments of cellular uptake and efflux across the apical membrane. For this purpose, LLC-PK1 cells were grown on 12-Transwell plates in order to block the basal side of the cell. The uptake of TBuMA reached a steady state 15 min after the start of the incubation (data not shown). In the present study, the amount of TBuMA in the cell at 30 min was measured. The uptake amount of TBuMA increased by 8.5-fold as the concentration of TBuMA in the transport medium was increased 10-fold from 10 μM to 100 μM, suggesting a saturation in the uptake process. The apical uptake of TBuMA (10 and 100 μM) decreased significantly in the presence of TDC on the apical side (32 and 31% decrease for 10 and 100 μM TDC, respectively (Fig. 5A), demonstrating decreased uptake for the TBuMA-TDC ion-pair complex.

The efflux of TBuMA across the apical membrane from the cell preloaded with the ion-pair complex was monitored for a 2-min period. The efflux of TBuMA was rapid, reaching equilibrium in 2 min (Fig. 5B). The temporal efflux and the efflux rate of TBuMA, estimated using the data for the first 1 min period (Fig.5C) were decreased significantly by the presence of representative P-gp substrates, daunomycin and verapamil (all 100 μM), in the transport medium, but not by the

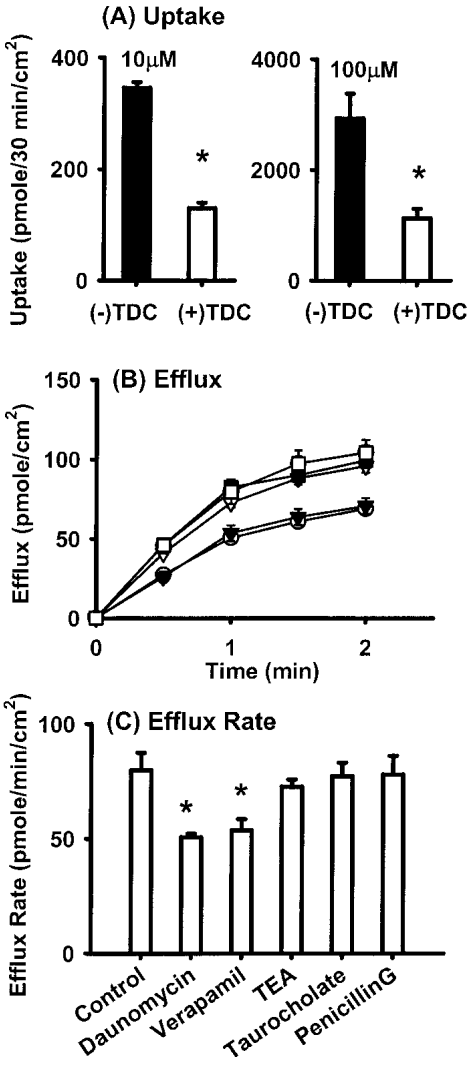

Fig. 5. Cellular uptake (A) of 10 and 100 μM TBuMA after incubation for 30 min in the presence and absence of 10-fold molar excess of TDC at pH 7.4 across the apical membrane of cells, and the effect of various compounds (100 μM each, ●; control, ○; daunomycin, ▼; verapamil, ▽; TEA, ■; taurocholate, and □; penicillin G) on the efflux (B, C) of TBuMA-TDC complex across the apical membrane of cells. The efflux was measured after preloading the cells with 100 μM TBuMA and 1mM TDC for 30 min at pH 7.4, and the efflux rate of TBuMA-TDC complex in the presence of various compounds (100 μM each) was calculated from the linear portion (up to 1 min) of the efflux profile. Closed symbols indicate the cellular uptake of TBuMA only, and open symbols the cellular uptake and efflux of the TBuMA-TDC complex. Data represent the mean± SD of three determinations. *$p < 0.01$ compared with the (−) TDC (A) and control (C) conditions by unpaired Student *t* test.

presence of TEA, taurocholate and penicillin G (all 100 μM). The above results indicate that P-gp is involved in the apical efflux of TBuMA in the presence of TDC (i.e., the efflux of the ion-pair complex). The decrease in the amount of TBuMA taken up in the presence of TDC (Fig. 5A) may be a reflection of the increase in the efflux of the ion-pair complex via the P-gp.

DISCUSSION

In the presence of a proton gradient across the cell monolayer (i.e., pH 6.0 apical/pH 7.4 basal), the B-A transport of TBuMA and TEMA were significantly higher than the corresponding A-B transport, and the kinetic parameters (K_m and V_{max}) for the B-A transport of these OCs were comparable to those of a representative substrate of the OC/H^+ antiporter (i.e., TEA), consistent with the operation of the OC/H^+ antiport system for the B-A transport of these OCs (9,10,16). The B-A transport of these OCs (10 μM) was not influenced by the presence of TDC (100 μM; Fig. 2A), contrary to an expectation that TDC might increase the passive diffusion of TBuMA via the formation of a lipophilic ion-pair complex with TBuMA. The reduced affinity of the compound to the responsible transporter, due to the masking of the positive charge of TBuMA through the formation of lipophilic ion-pair complexes (1,17), might have cancelled out the increased passive diffusion of the compound. Regardless of the mechanisms involved, the above results imply that the formation of ion-pair complexes in the kidney has little physiologic relevance to the urinary excretion of these OCs, in a situation where a proton gradient exists between the plasma and urine (i.e., acidic urine compared to plasma).

In general, the pH of urine is acidic compared to the plasma, thus permiting the establishment of a proton gradient. However, the pH of urine often varies widely (5.0–8.0), often yielding a situation where no significant pH difference between the plasma and urine exists (18,19). Thus, the transport of OCs in such a case was investigated. Under proton equilibrated conditions (i.e., pH 7.4 apical/pH 7.4 basal, for example), the B-A transport of TEMA, but not the A-B transport, was reduced significantly to a level comparable to the A-B transport (Fig. 2B), consistent with the hypothesis that the OC/H^+ antiporter is no longer operative under proton equilibrated conditions. TDC had no influence on the transport of TEMA in either direction (Fig. 2B), consistent with the observation that TEMA does not form lipophilic ion-pair complexes in the presence of bile salts (4,5). Therefore, a substantial decrease in the renal excretion of TEMA would be expected as the proton gradient between the plasma and urine disappears. In fact, the renal clearance of TEA, a homologue of TEMA, was decreased significantly (61%), when the pH of urine in rats was intentionally elevated to 7.1 (19).

The transport of TBuMA, in either direction, was also significantly reduced under pH-equilibrated conditions. Nevertheless, the B-A transport was maintained at a much higher level compared to A-B transport (Fig. 2B), indicating that an efflux system distinct from the OC/H^+ antiport system is operative under these conditions. Most interestingly, the B-A transport of TBuMA, but not the A-B transport, was significantly increased in the presence of TDC (Fig. 2B), consistent with the formation of lipophilic ion-pair complexes of TBuMA, followed by an acceleration in excretion via the responsible transporter under pH equilibrated conditions (4). A kinetic analysis revealed that TDC increased the affinity of TBuMA to the relevant transporter by 2.2-fold (as evidenced by a 54% decrease in the K_m value; Table I), but not for the capacity of the transport (as evidenced by the constant V_{max} value; Table I), resulting in a 2.4-fold increase in the intrinsic clearance (CL_{int}) of TBuMA (Table I). A slight increase in passive diffusion in the B-A direction was also observed (Table I). The above results imply that the formation of ion-pair complexes in the kidney, probably with bile salts, might influence the *in vivo* renal excretion of large Mw Ocs, such as TBuMA, in a situation where no pH gradient exists between the plasma and urine.

Under proton gradient conditions, a 2.5-fold larger total clearance (i.e., sum of CL_{int} and CL_{linear}) was obtained for TEMA compared with TBuMA, consistent, in part, with the slightly larger *in vivo* renal clearance of TEMA compared to TBuMA (3). Under pH-equilibrated conditions, on the contrary, more than a 4.1-fold larger total clearance was obtained for TBuMA compared with TEMA (Table I). Moreover, the total clearance of TBuMA, under pH equilibrated conditions, was further increased by the presence of TDC, yielding a 9.2-fold difference from that of TEMA (Table I). Thus, a larger *in vivo* renal clearance would be expected for TBuMA compared to TEMA, in a situation where no proton gradient exists between the plasma and urine, and the formation of ion-pair complexes with bile salts in the kidney is assumed. The *in vivo* renal excretion of TBuMA and TEMA under pH-equilibrated condition is under investigation in rats.

The vectorial transport of TBuMA in the presence of TDC, under pH-equilibrated conditions, was influenced by the presence of representative P-gp substrates such as daunomycin, verapamil and cyclosporine A, resulting in a decreased B-A transport, and correspondingly increased A-B transport (Fig. 4A). In a retrograde, the transport of daunomycin was influenced by the presence of the ion-pair complex (Fig. 4B). Thus, the involvement of P-gp in the transport of the ion-pair complex is indicated, consistent with a previous report (4). The release of the TBuMA-TDC ion-pair complex across the apical membrane of the LLC-PK1 cell (i.e., efflux) was decreased by the presence of P-gp substrates in the apical side (Fig. 5B), confirming the involvement of P-gp.

In conclusion, the B-A transport of TBuMA was significantly increased, under pH equilibrated situation (pH 7.4 apical/pH 7.4 basal, for example), in the presence of TDC, probably as the result of the formation of a lipophilic ion-pair complex, and the increase in the affinity to P-gp appears to be the responsible mechanism. A summary of the proposed mechanism is shown as a scheme in Fig. 6. This reaction (i.e, ion-pair formation with endogenous bile salts) might accelerate the urinary excretion of large Mw OCs, in the pH equilibrated situation (i.e., no proton gradient between the plasma and urine). Under proton gradient conditions, however, ion-pair formation appears to have no significant physiologic relevance, since an OC/H^+ antiporter dominates the B-A transport of these OCs in such a case. Considering that the pH equilibration (18,19) and the presence of bile salts in the kidney (7,8) are highly likely, an acceleration in urinary excretion of large Mw OCs seems highly feasible through this interaction. Under pH equilibrated conditions, larger renal clearances would be expected for large Mw OCs, based on their

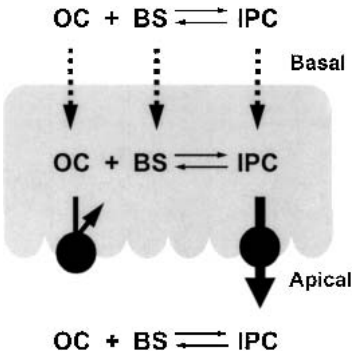

Fig. 6. Schematic illustration of a proposed model of the basal to apical transport of TBuMA across LLC-PK1 cell monolayers under the pH equilibriated condition (i.e., pH 7.4 apical/pH 7.4 basal). TBuMA represents large Mw organic cations (OCs) that form lipophilic ion-pair complexes (IPC) with endogenous bile salts (BS). Under this condition, the transport of large Mw OCs is accelerated in the presence of BS such as taurodeoxycholate (TDC) through forming ion-pair complexes (IPCs) with BS in the blood pool and/or in the kidney. Subsequently increased binding of the complexes to P-gp on the apical membrane appears to be a probable mechanism of the acceleration. The OC/H^+ antiport system does not appear to be operative. Only solid arrows in the scheme summarize the results of the present study.

greater tendency to form lipophilic ion-pair complexes with bile salts, compared to small Mw OCs (4,5). Conjugates of deoxy bile acids such as TDC, glycodeoxycholate, taurochenodeoxycholate and glycochenodeoxycholate appear to represent endogenous bile salts that form lipophilic ion-pair complexes with large Mw OCs (4). Ion-pair formation with these bile salts in the kidney might constitute a self-protection mechanism of the body against xenobiotics, in a special situation where proton equilibration conditions prevail, through accelerating the excretion of these compounds. This aspect should be carefully taken into account in attempts to explain the urinary excretion of OCs. However, it should be reminded that hydrophobic compounds are somewhat reabsorbed from the renal tubule, possibly offsetting the significance of OC-bile salt complexation in the renal excretion of high Mw OCs, even under pH equilibrated conditions between urine and plasma. Thus, *in vivo* evidences are needed before a conclusion on the physiologic relevance of ion-pair complexation is made.

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