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Mechanism of Intestinal Transport of an Organic Cation, Tributylmethylammonium in Caco-2 Cell Monolayers

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Many quaternary ammonium salts are incompletely absorbed after their oral administration and may also be actively secreted into the intestine. However, the underlying mechanism(s) that control the transport of these cations across the intestinal epithelium is not well understood. In this study, the mechanism of absorption of quaternary ammonium salts was investigated using Caco-2 cell monolayers, a human colon carcinoma cell line. Tributylmethylammonium (TBuMA) was used as a model quaternary ammonium salts. When TBuMA was administrated at a dose of 13.3 imole/kg via iv and oral routes, the AUC values were 783.7 ± 43.6 and 249.1±28.0 μmole·min/L for iv and oral administration, indicating a lower oral bioavailability of TBuMA (35.6%). The apparent permeability across Caco-2 monolayers from the basal to the apical side was 1.3 times (p<0.05) greater than that from the apical to the basal side, indicating a net secretion of TBuMA in the intestine. This secretion appeared to be responsible for the low oral bioavailability of the compound, probably mediated by p-gp (p-glycoprotein) located in the apical membrane. In addition, the uptake of TBuMA by the apical membrane showed a Na^+ dependency. Thus, TBuMA appears to absorbed via a Na^+ dependent carrier and is then secreted via p-gp related carriers.

Key words: Quaternary ammoniums, TBuMA, Caco-2, P-gp

INTRODUCTION

The small intestine is an important site of absorption for orally administered drugs. It also serves as an important route for the clearance of xenobiotics and endogenous metabolites (Israili and Dayton, 1984) in concert with their metabolism and elimination by the liver and kidney. It has long been assumed that the intestinal absorption of drugs after oral administration is mediated by a simple diffusion process, which is dependent on the physicochemical properties of the compound such as hydrophobicity and ionization state. However, a number of drugs have been reported to show higher or lower absorption rates after their oral administration than would be expected from their physicochemical properties. In the 1980's, the development of *in vitro* experimental techniques such as isolated membrane vesicles, the Ussing chamber mounted with the intestine, and Caco-2 cell culture systems have allowed the transport mechanisms of various drugs across the membrane of the small intestine to be elucidated (Murer *et al.*, 1989; Hidalgo *et al.*, 1989).

Extensive surveys concerning the mechanisms of intestinal absorption for various ionic drugs have revealed that drug transporters can be largely classified into three systems; organic cation transport systems, organic anion transport systems and peptide transport systems (Katsura and Inui, 2003). However, less attention had been paid to the transport of organic cations in the small intestine (Koepsell *et al.*, 2003).

A variety of drugs and endogenous bioactive amines are organic cations (OCs). In fact, approximately 40% of all conventional drugs on the market are OCs, as exemplified by anisotropine, isopropamide, diphenamyl, methanthline, atracurium, pancuronium, bretylium, and glycopyrrolate (Neuhoff *et al.*, 2003). Thus, the transport of xenobiotics or endogenous OCs in the body has been a subject of considerable attention since a family of OC transporters (OCTs, Gorboulez *et al.*, 1997; Zhang *et al.*, 1997), and a new subfamily of OCTs (Yabuuchi *et al.*, 1999) and

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OCTNs (Wu *et al.*, 1999), have been cloned, leading to the functional characterization of these transporters in various systems including oocytes and cell lines.

TBuMA is frequently used as a model OC based on the absence of any significant metabolism in the body and the absence of plasma protein binding (Neef *et al.*, 1984). In our previous study, the oral bioavailability of tributylmethyl ammonium (TBuMA, MW. 200), a model OC, was found to be less than 17.1~35.0% for 0.4~12 μmole/kg dose in rats (Kim *et al.*, 2005). This discrepancy was addressed by a mechanism related to the efflux transport systems on the apical membrane (e.g. p-gp) by Ussing chamber studies. The objective of this study, therefore, was to investigate the mechanisms that control the intestinal transport of an organic cation, i.e. TBuMA, using Caco-2 cell monolayers.

MATERIALS AND METHODS

Materials

[^{3}H]TBuMA (specific activity, 1.0 Ci/mmol) was synthesized according to the method of Neef *et al.* (1984), by the reaction of an excess of the corresponding tertiary amines with [^{3}H]methyliodide (Amersham, Arlington Heights, IL; specific activity, 85 Ci/mmole). All other reagents employed were of the highest grade commercially available. [^{14}C] mannitol (50 mCi/mmole, New England Nuclear, Boston, MA), fetal bovine serum (Hyclone Laboratories, Logan, UT), trypsin-EDTA (Gibco Laboratories, Gaithersburg, MD), and Dulbecco's modified Eagle's medium, nonessential amino acid solution, penicillin/streptomycin, Hank's balanced salt solution (HBSS), HEPES (all from Sigma Chemical Co.) were used as purchased. All other reagents were of analytical grade.

Systemic pharmacokinetic study

Under light ether anesthesia, the femoral artery and vein of rats were cannulated with polyethylene tubing (PE-50) for blood sampling and the administration of TBuMA, respectively. After recovery from the surgery, a saline solution of [^{3}H]TBuMA was administered via both intravenous (iv) and oral (po) routes to rats at the dose of 13.3 μmole (13.2 μCi)/kg.

Blood samples were collected at appropriate intervals over a 24 h period, and each plasma sample was isolated by centrifuging the blood at 13,000 rpm for 5 min. The concentration of [^{3}H]TBuMA in the plasma was quantified by means of liquid scintillation counting (LSC System 1409, Wallac, Finland). All counts in the plasma samples were to be considered significant only when they exhibited more than five-fold higher values compared to a blank plasma sample. The area under the plasma concentration-time curve from time zero to infinity ($AUC_{0-\infty}$) was calculated by the trapezoidal rule, followed by extrapolation. Bioavailability was calculated from the ratio of $AUC_{0-\infty}$ for oral administration versus iv administration.

Cell cultures

The human colon adenocarcinoma cell line, Caco-2 (American Type Culture Collection, Rockville, MD), was grown in the form of monolayers in Dulbecco's modified Eagles' medium, 10% fetal bovine serum, 1% nonessential amino acid solution, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. Stock cultures were grown in 75-cm^2 tissue culture flasks and were split 1:3 at 80 to 90% confluency using 0.02% EDTA and 0.05% trypsin. The Caco-2 cells from passage numbers 46 to 55 were seeded on permeable polycarbonate inserts (1 cm^2, 0.4 μm pore size; Corning Costar Co., Cambridge, MA) in 12 Transwell plates (Corning Costar Co.) at a density of 2.5×10^5 to 3.0×10^5 cells/cm^2. The inserts were fed with the incubation medium at 2-day intervals for the first week and then at daily intervals, usually for 2 weeks, until they were used in the transport experiments. The integrity of the cell monolayers was evaluated by means of transepithelial electrical resistance (TEER) measurements using an EVOM™ epithelial volt/ohm-meter (World Precision Instruments, Sarasota, FL). When the TEER value reached 300-700 Ωcm^2, the cell inserts were used in the transport experiments. The transport of [^{14}C]mannitol (5.4 μM) was <0.25% of the dose/h, corresponding to an apparent permeability (P_{app}) value of 5.6×10^7 cm/s under the culture conditions used.

Transport study

Before the transport experiments, the cell monolayers were washed three times with transport medium (pH 7.4, HBSS containing 25 mM HEPES and 25 mM glucose). After each wash, the plates were incubated in transport medium for 1 h at 37°C, and the TEER value was then measured. The transport medium on both sides of the cell monolayers was then removed by aspiration. Three inserts were used in each transport experiment. To measure the transport of TBuMA (1 μM) from the apical-to-basal (A-B) side, 0.5 mL of transport medium (pH 7.4) containing the drug was added to the apical side, and 1.5 mL of transport medium without the drug was added to the basal side. The inserts were moved to wells containing fresh transport medium at 30 min intervals over a 2 h period. A 300 μL aliquot of the medium, taken at each time point, was assayed for TBuMA by liquid scintillation counting (LSC). For measurement of the basal-to-apical (B-A) transport of TBuMA, 1.5 mL of transport medium containing the drug was added to the basal side, and 0.5 mL of transport medium without the drug was added to the apical side.

The inserts were then incubated at 37°C, and 300 μL aliquots were removed from the apical side at 30 min intervals over a 2 h period and replaced with 300 mL of fresh transport medium. A 300 mL aliquot of each sample was assayed for TBuMA by LSC. In experiments to investigate the effect of various compounds on the B-A transport of 1 μM TBuMA, inhibitors were added to the transport medium on the same side of the cell monolayers. In addition, the effect of a Na^+ gradient on the uptake of TBuMA across the apical membrane into Caco-2 epithelial cells was investigated in the presence or absence of Na^+ in the transport medium. The levels of [^{3}H]TBuMA and [^{14}C]mannitol were determined by liquid scintillation counting using a Wallac model 1409 instrument (Wallac, Gaithersburg, MD).

Calculation

For each transport experiment, the mean transport rate was calculated from the linear portion (i.e., 30-, 60-, 90-, and 120-min time points) of a plot of the total amount of drug transported versus time. The apparent permeability values, P_{app}, of drug across the Caco-2 cell monolayers, expressed as cm/s, were calculated using the following equation,

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A} \cdot \frac{1}{C_o} \quad (1)$$

where dQ/dt is the permeability rate (μmole/min), A the surface area of the membrane (cm^2, 1 in the present study), and C_o the initial concentration of the drug in the donor chamber (μmole/mL). All data are expressed as the mean ± SD of three experiments. The statistical significance of differences between treatments was evaluated using unpaired Student's *t* tests, and a value of $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Systemic pharmacokinetics of TBuMA

Temporal profiles for the plasma concentration of TBuMA after intravenous and oral administration to rats at a dose of 13.3 μmole/kg are shown in Fig. 1. The plasma concentrations of TBuMA following oral administration reached peak values at the second sampling time point (5 min), indicating that the gastrointestinal absorption of TBuMA was extremely rapid, and followed a triexponential decline. The mean half-lives of TBuMA at the terminal phase were extremely long (more than 4 h), in agreement with previous reports (Kim *et al.*, 2005; Hong *et al.*, 2000; Han *et al.*, 1999; Neef *et al.*, 1984). When TBuMA was administrated at a dose of 13.3 μmole/kg *via* iv and oral routes, the AUC values were 783.7 ± 43.6 and 249.1 ± 28.0 μmole·min/L for iv and oral administrations, indicating a lower oral bioavailability of TBuMA (35.6%).

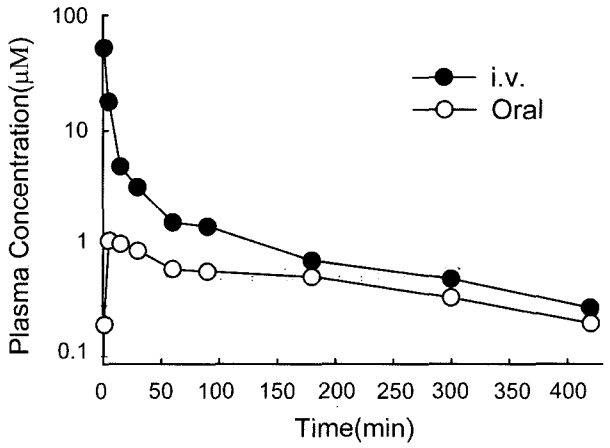

Fig. 1. Time-plasma concentration profiles for TBuMA in rats following intravenous (iv) and oral (po) administration at a dose of 13.3 μmole/kg. Each data point represents the mean ± S.D. of four animals (●, iv; ○, oral).

Transepithelial transport of TBuMA in Caco-2 cells

To examine the issue of whether carrier-mediated transport is involved in the transepithelial transport of TBuMA across Caco-2 cell monolayers, the directional transport of TBuMA was determined as a function of time (Fig. 2A). The polarized transport of TBuMA was observed to be much faster in the B-A direction compared with the A-B direction. The apparent permeability coefficient of TBuMA in the B-A direction was significantly greater compared with the A-B direction for TBuMA (1 μM). For example, the permeability coefficient was approximately 37% greater for the B-A flux (2.2×10^{-6} cm/s) compared with the A-B flux (1.6×10^{-6} cm/s) for TBuMA (1 μM, Fig. 2B), suggesting the involvement of a carrier-mediated system for the B-A transport of TBuMA in Caco-2 cells.

Effects of the modulators on transport of TBuMA in Caco-2 cells

It is well known that p-gp is expressed in the apical membrane of mucosal cells in the intestine and plays a role in pumping (i.e., secretion) a variety of xenobiotics into the lumen, leading to a limited net intestinal absorption of such compounds. Thus, the relationship between the active transport of TBuMA in the efflux (secretary) direction across the Caco-2 cell monolayers and p-gp was examined. For this purpose, the flux of TBuMA (1 μM) was examined in the absence and presence of representative P-gp inhibitors such as verapamil (100 μM), cyclosporine A (100 μM) on the apical and basal sides. The presence of the above p-gp inhibitors had no significant effect on the TEER values for the Caco-2 cell monolayers, but substantially increased the A-B, and inhibited the B-A flux of TBuMA (Fig. 4), which

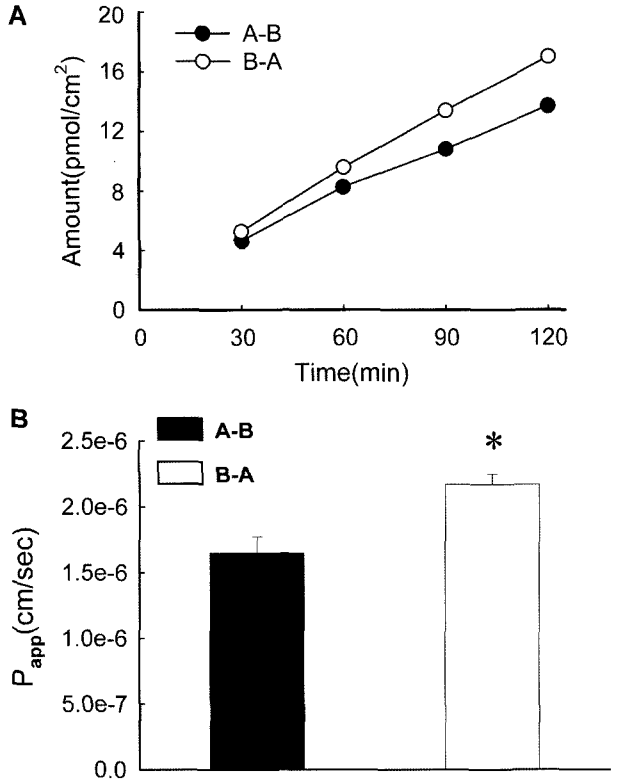

Fig. 2. Time course **(A)** of the apical-to-basal (A-B, ●) and basal-to-apical transport (B-A, ○) of 1 μM TBuMA (mean ± S.D., n =3) across Caco-2 cell monolayers at 37°C, and its permeability (cm/sec, **B**)

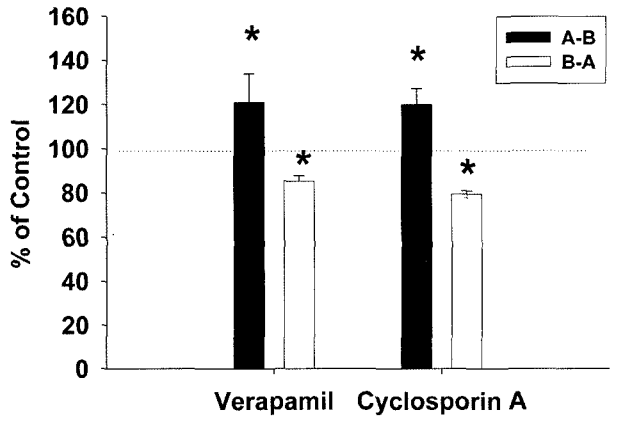

Fig. 3. Effect of P-gp inhibitors (verapamil, cyclosporine A, 100 μM) on the transport of TBuMA (1 μM) across Caco-2 cell monolayers. The values of the y-axis are presented as the percentage of the control values (TBuMA only) transported in the A-B (closed bars) and B-A (open bars) directions. Each data point represents the mean ± S.D. of three different preparations.

was also found in our previous study using a Ussing chamber mounted with rat intestines. This suggests that TBuMA is transported across Caco-2 cell monolayers *via* p-gp, and that an active carrier-mediated active mechanism is operative.

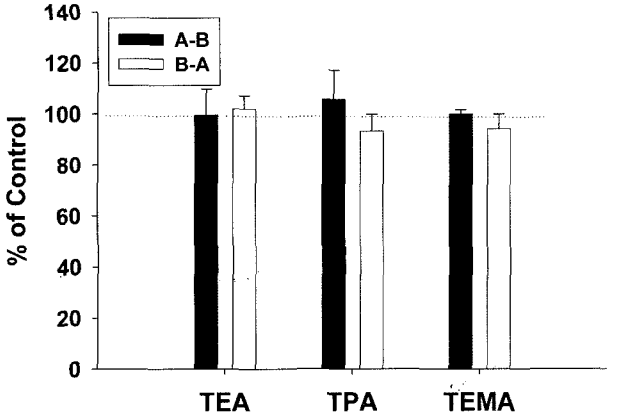

Fig. 4. Effect of organic cations (100 μM) on the transport of TBuMA (1 μM) across Caco-2 cell monolayers (TEA, tetraethylammonium; TPA, tetrapropylammonium; TEMA, triethylmethyl-ammonium). The values of the y-axis are presented as the percentage of the control values (TBuMA only) transported in A-B (closed bars) and B-A (open bars) directions. Each data point represents the mean ± S.D. of three different preparations.

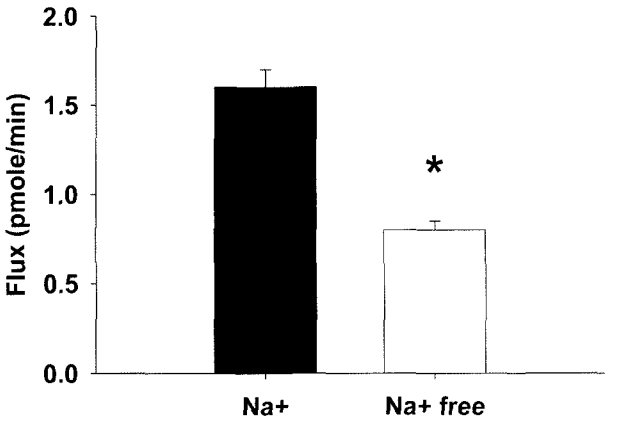

Fig. 5. Effect of a Na^+-potential gradient on the transport of TBuMA (1 μM) across Caco-2 cell monolayers. Each data point represents the mean ± S.D. of three different preparations.

However, the presence of structurally similar OCs, tetraethylammonium (100 μM), tetrapropylammonium (100 μM), triethylmethylammonium (100 μM) had no effect on either the A-B or B-A transport of TBuMA (1 μM) (Fig. 4), suggesting that the transport system for TBuMA is independent of other transport systems that are involved in the transport of other organic cations.

In order to investigate the involvement of potential-driven transporters in the uptake transport of TBuMA, the uptake of TBuMA into cells was determined in the presence or absence of a Na^+ potential gradient. As a result of the depolarization, the uptake flux of TBuMA was significantly reduced by 50% (Fig. 5). Even if a carrier or transporter responsible for the absorption of TBuMA in the intestine was not specifically identified in this study, it can safely be

assumed that TBuMA is transported into epithelial cells *via* a Na^+ dependent carrier. In future studies, the nature of the specific transporter related to the transport of TBuMA will need to be investigated.

CONCLUSION

The mechanism of absorption of quaternary ammonium salts was investigated in rats and Caco-2 cell monolayers, a human colon carcinoma cell line. When tributylmethylammonium (TBuMA) was administered to rats, the oral bioavailability of TBuMA was calculated to be 35.6%. The low bioavailability was concluded to be due to the lower permeability from the basal to the apical side across the monolayer compared to that from the apical to the basal side. The secretion of TBuMA appeared to be mediated by p-gp (p-glycoprotein) located in the apical membrane, and TBuMA was assumed to be transported into intestinal epithelial cells via a Na^+ dependent carrier.

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