Different Activity of ATP Dependent Transport Across the CanaliculAr Membrane for TributylmethyLammonium and TriethylmethyLammonium as a Potential Mechanism of the Preferential Biliary Excretion for TributylmethyLammonium in the Rat

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Purpose. The mechanism(s) responsible for the significantly higher biliary excretion of tributyl methyl ammonium (TBuMA) than of triethyl methyl ammonium (TEMA) was investigated in canaliculAr liver plasma membrane vesicles (cLPM).

Methods. The uptake of [3H]TBuMA and [3H]TEMA into cLPM in the presence of a pH gradient or ATP was measured by a rapid filtration technique.

Results. The uptake of substrates into the vesicle was significantly increased by an outwardly directed pH gradient. The pH dependent uptake was saturable and cross-inhibited by the other organic cation, indicating that TEMA and TBuMA share a common transport mechanism. Kinetic analysis revealed the two compounds show similar characteristics for the pH-gradient dependent uptake. Thus, the organic cation/H+ exchange mechanism does not appear to explain the significant difference in biliary excretion of the organic cations. In the presence of ATP, however, uptake into cLPM was readily observed for TBuMA while TEMA uptake was negligible. Inhibition studies with typical P-glycoprotein substrates indicated the uptake may be mediated by the P-glycoprotein.

Conclusions. Differences between TBuMA and TEMA in reactivity for an ATP dependent transport process, rather than for an organic cation/H+ exchanger, may be responsible for the markedly different biliary excretion of TBuMA and TEMA.

KEY WORDS: TBuMA; TEMA; cLPM; biliary excretion; P-glycoprotein; organic cation/H+ exchanger.

INTRODUCTION

The extent of biliary excretion of organic cations (OCs) varies depending on the molecular weight (Mw) of the compounds (1,2). For example, tributyl methyl ammonium (TBuMA, Mw 200) is primarily excreted (in excess of 40% of dose) into the bile of rats whereas, only negligible excretion (about 0.3%) is observed for triethyl methyl ammonium (TEMA, Mw 116, 3). Currently, the underlying mechanism(s) for differences such as these is (are) unknown. In a previous study (4) we demonstrated the canaliculAr membrane transport process appears to be responsible for these differences. Since a number of carrier mediated mechanisms in the liver canaliculAr membrane have been suggested to participate in OC transport (5–11), it is logical to hypothesize these transporters may be involved in the marked variations in OC excretion into the bile. Among these transport systems, an OCH+ exchanger has been the most intensively studied. For example, the antipporter was reported to transport TBuMA (6) and tetraethyl ammonium (TEA, 5), a structural analog of TEMA. However, it was not clear whether this transport mechanism is responsible for the marked difference in biliary excretion of TBuMA and TEMA.

Recently, Smit et al. reported a P-glycoprotein (P–gp) mediated transport of some OCs including TBuMA in LLC-PK1 cell line in which P–gp was overexpressed by transfection of mdr1a and mdr1b genes (11). They also reported significantly decreased biliary excretion of TBuMA in mdr1a and mdr1a/1b gene knockout mice (12,13). Therefore, it is reasonable to suspect that the P–gp transport system in the liver canaliculAr membrane may also be involved in the biliary excretion of TBuMA, in addition to the OCH+ exchanger. However, P–gp mediated transport has never been examined for the presence of TEMA.

Therefore, the objective of this study is to identify the transport mechanism in canaliculAr liver plasma membrane vesicles (cLPM) responsible for the marked difference in biliary excretion of TBuMA and TEMA. Thus, we compared transport activity of OCH+ exchanger for TBuMA and TEMA. In addition, we were interested in examining whether the P–gp mediated transport mechanism in the liver could be demonstrated for the OCs and determining whether the transport system contributes to the preferential transport of TBuMA across the canaliculAr membrane.

MATERIALS AND METHODS

Chemicals

[3H]TBuMA (0.2 Ci/mmol) and [3H]TEMA (0.2 Ci/mmol) were synthesized according to the method of Neef et al. (14). [3H]Methyl iodide (85 Ci/mmol) was purchased from American (Arlington Heights, Illinois). Unlabelled methyl iodide, triethylamine, and tributylamine were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). [3H]Taurine (3.7 Ci/mmol) and [3H]daunomycin (4.4 Ci/mmol) were purchased from New Life Science products (Boston, Massachusetts). Unlabelled daunomycin was a gift from Dong-A Pharm Co. (Seoul, Korea). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Preparation of CanaliculAr Liver Plasma Membrane Vesicles

CanaliculAr liver plasma membrane vesicles (cLPM) were prepared from male SPF SD rats (Dae-Han Experimental Animal, Seoul, Korea; 250–270 g in body weight) by the method of Inoue et al. (15). The activity of alkaline phosphatase (16), a marker enzyme for canaliculAr membrane, was enriched approximately 5-fold in the vesicle preparation, compared with that in crude liver homogenates. Protein concentration of the vesicle preparation was 0.14 ± 0.03

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mg/g of liver when measured by the Lowry method (17) using bovine serum albumin as a standard. The inside-out proportion of vesicles was more than 30% (i.e., 69.8 ± 8.6% for right-side-out vesicles, mean ± SD for four measurements) when determined by measurement of exposed sialic acid concentration (18), which is consistent with the previous reports (19,20). Functional activity of the vesicle preparation was confirmed as evidenced by at least a 5-fold higher uptake of [3H]taurocholate in the presence of ATP regenerating system compared with the uptake in the absence of an ATP (21,22). Immediately after the preparation, the cLPm was suspended in a membrane suspension buffer (MSB) containing (in mM) 250 sucrose, 10 Hepes, 10 Tris, 10 MgCl₂, and 0.2 CaCl₂ (pH 7.4) to yield a protein concentration of 4–6 mg/ml. The suspension was stored at 70°C for up to 2 weeks until transport studies were carried out.

Vesicle Uptake Studies

The uptake of [3H]TBuMA and [3H]TEMA into cLPm vesicles in the presence of a pH gradient or ATP was measured by a rapid filtration technique (23). A frozen suspension was quickly thawed by immersing it into a 37°C water bath, revesiculated by passing it 20 times through a 25-gauge needle, and diluted with MSB to give 1–1.5 mg/ml of protein. For 4 min 20 μl of the diluted suspension was preincubated in a test tube at 37°C. Then, 80 μl of an incubation buffer, containing 5 μM [3H]TBuMA or [3H]TEMA (0.080 μCi each), was added to the diluted vesicle suspension. In studies involving pH gradient dependent uptake, buffers containing (in mM) 82 sucrose, 100 K⁺-glucuronate, 91 MES, 14 Hepes, 29 Tris, 0.2 Ca-glucuronate (for pH 5.9 conditions), and 70 sucrose, 100 K⁺-glucuronate, 76 Hepes, 70 Tris, 0.2 Ca-glucuronate (for pH 7.9 conditions) were used. MSB (pH 7.4) was used as an incubation buffer for ATP dependency studies. After predetermined time intervals, the uptake was quenched by the addition of 4 ml of ice-cold stop solution, which consisted of (in mM) 250 sucrose, 10 Hepes, 10 Tris, 10 MgCl₂, 0.2 CaCl₂, 1 TBuMA, or TEMA (pH 7.4, for ATP dependency studies), 204 sucrose, 150 K⁺-glucuronate, 10 Hepes, 10 Tris, 5 Mg-glucuronate, 0.2 Ca-glucuronate, 1 TBuMA, or TEMA (pH 7.5, for uptake studies involving pH gradient). The entire contents were then rapidly filtered through a 0.45 μm MF-MEMB 25 mm filter (Seoul Science, Seoul), which was presoaked 2 h in ice cold stop solution. The tube was rinsed again with 4 ml of respective ice cold stop solution and filtered. After washing twice with 4 ml of the ice cold stop solution, the filter was dissolved in 4 ml of a scintillation cocktail (Ultima Gold, Packard, Meriden, Connecticut), and the radioactivity of the mixture was measured using a Wallac 1409 liquid scintillation counter (Wallac, Turku). Presoaking and rinsing the filter with the ice cold stop solution, which contains 1 mM TBuMA or TEMA, resulted in almost negligible nonspecific binding of OCs to the filter (i.e., negligible radioactivity in the filter; data not shown). The binding of TEMA or TBuMA to the surface of vesicles at equilibrium (i.e., 60 min) was approximately 15% of the peak uptake value, when it was estimated from the relationship between the uptake and the osmolarity of the incubation medium. Thus, the estimated transport is not likely to be affected by the presence of bound OCs to the surface. When it was necessary to examine the concentration dependency for the uptake, OC uptake for 30 sec was examined for 0.01–5 mM TBuMA and TEMA (while fixing the [3H] specific activity at 0.16 μCi/μM for each substrate) in the presence of a pH gradient or ATP. The effect of representative P-gp substrates (50 μM), OCs (100 μM), and an organic anion (benzyl penicillin, 100 μM) on a 30 sec period of uptake of 10 μM [3H]TBuMA (0.16 μCi) was also measured in the presence

![Fig. 1. Uptake of OCs (5 μM) into cLPm in the presence (●) and absence (○) of an outwardly directed pH gradient for TBuMA (A) and TEMA (B). Membrane vesicles were treated with valinomycin (5 μg/ml protein) for 10 min at 37°C. Each data point is expressed as means ± S.D. of triplicate measurements for three different batches of cLPm. * p < 0.01 from two-way ANOVA.](image)

<p>| Table I. Inhibitory Effect of OCs on TBuMA Uptake into cLPm in the Presence of a pH Gradient* |
|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration</th>
<th>Pmole/mg protein/30s</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>239.7 ± 11.2</td>
<td>—</td>
</tr>
<tr>
<td>TBuMA</td>
<td>100 μM</td>
<td>118.7 ± 9.0*</td>
<td>49.4</td>
</tr>
<tr>
<td>TPMA</td>
<td>100 μM</td>
<td>122.3 ± 10.7*</td>
<td>51.0</td>
</tr>
<tr>
<td>TEMA</td>
<td>100 μM</td>
<td>133.3 ± 6.7*</td>
<td>55.6</td>
</tr>
</tbody>
</table>

* Each data point is expressed as means ± S.D. of triplicate measurements of three batches of membrane vesicle preparations.

* p < 0.01 from Student’s t-test.