Correlation between apical localization of Abcc2/Mrp2 and phosphorylation status of ezrin in rat intestine

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Abbreviations: ABC transporter, ATP-binding cassette transporter; Mrp2/Abcc2, multidrug resistance-associated protein 2/ATP-binding cassette transporter family C2; P-gp/Mdr1/Abcb1, P-glycoprotein/multidrug resistance protein 1/ATP-binding cassette transporter family B1; Bcrp/Abcg2, breast cancer resistant protein/ ATP-binding cassette transporter family G2; Thx, thymeleatoxin; PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; BBM, brush border membrane; ERM, (ezrin/radixin/moesin); mKRBB, modified Krebs-Ringer bicarbonate buffer; MCB, monochlorobimane; GS-B, glutathione S-bimane; EHBR, Eisai hyperbilirubinemic rat
Abstract

The multidrug resistance-associated protein 2/ATP-binding cassette transporter family C2 (Mrp2/Abcc2) is an ATP-dependent export pump that mediates the transport of a variety of organic anions. Abcc2 is mainly expressed on the canalicular membrane of hepatocytes and also the brush border membrane of intestinal epithelial cells. We have previously reported that Abcc2 is rapidly internalized from the canalicular membrane during acute oxidative stress, which induces protein kinase C (PKC) activation in rat liver. However, it has not been elucidated whether PKC is involved in the regulation of Abcc2 localization in other tissues. In this study, we investigated this issue in rat intestinal epithelia. Exposure to thymeleatoxin, a conventional PKC (cPKC) activator, for 20 min reduced the cumulative glutathione S-bimane efflux for 40 min via Abcc2 from 30.3 ± 2.1 nmol/cm to 18.1 ± 1.6 nmol/cm. Likewise, the Abcc2 expression in the brush border membrane of the small intestine was reduced to half of that of the control without changing the total amount of Abcc2 present in the homogenate. Immunoprecipitation analysis suggested an interaction between Abcc2 and ezrin, a scaffolding protein that is dominantly expressed in the intestine. Thymeleatoxin treatment decreased the amount of the active form (C-terminally phosphorylated form) of ezrin as well as the amount of Abcc2 that co-immunoprecipitated with ezrin. These results indicate that cPKC activation diminishes the protein-protein interaction between ezrin and Abcc2. In conclusion, the phosphorylation status of ezrin correlates with the cell surface expression of Abcc2 in the rat small intestine, which may be regulated by cPKC.
Introduction

The small intestine is a highly differentiated organ with a barrier function against xenobiotics and a gateway function for nutrients. The ATP-binding cassette (ABC) transporter family, including P-glycoprotein/multidrug resistance protein 1 (P-gp/Mdr1/Abcb1), breast cancer resistant protein (Bcrp/Abcg2), and multidrug resistance-associated protein 2 (Mrp2/Abcc2), are well known efflux transporters that are located on the brush border membrane (BBM) of small intestinal epithelia to limit the absorption of a broad range of compounds (Takano et al., 2006). The distinct but sometimes overlapping substrate specificities of these efflux transporters have been demonstrated previously (Chan et al., 2004). Indeed, a significant increase in the oral absorption of their substrates including drugs and carcinogens was confirmed in animals genetically deficient in these transporters (Dietrich et al., 2001; Yamaguchi et al., 2002).

Several reports indicated the disparity between mRNA and protein expression of Abcc2 in the intestine. Naud et al. reported that Abcc2 protein expression was reduced to about 40% of that of the control during chronic renal failure in rats, but its mRNA expression was unaffected (Naud et al., 2007). Dietrich et al. also reported ABCC2 protein expression was reduced to about 27% of that of the control patients in the human intestine during obstructive cholestasis, but its mRNA expression was unaffected (Dietrich et al., 2004). Although the precise mechanism is unknown, these reports suggested the presence of post-transcriptional and/or post-translational regulation of intestinal ABCC2/Abcc2.

Post-translational regulation of Abcc2 is well defined in hepatocytes, where it is most abundantly expressed on the apical bile canalicular membrane. The stable expression of Abcc2 on the bile canalicular membrane requires radixin, a member of the ERM (ezrin / radixin / moesin) protein family that connects particular membrane proteins
to F-actin in the cytoskeleton (Kikuchi et al., 2002). Without radixin, membrane surface Abcc2 is selectively reduced, although that in the total cell lysate is minimally affected as demonstrated in radixin knockout mice and in isolated rat hepatocyte cultures that have had their radixin expression suppressed (Kikuchi et al., 2002; Wang et al., 2006). Moreover, Abcc2 is rapidly internalized from the bile canalicular membrane under various conditions, including oxidative stress (Ji et al., 2004), hyperosmotic perfusion (Kubitz et al., 1997), and ethynylestradiol administration (Mottino et al., 2002), probably as a result of post-translational regulation. Certain PKC family members sometimes play a role in the redistribution process of Abcc2 in hepatocytes. We have recently reported that the internalization of Abcc2 induced by oxidative stress is regulated by novel PKC (nPKC) in rat liver (Sekine et al., 2006), while Kubitz et al. reported that activation of PKC by phorbol ester caused redistribution of ABCC2/Abcc2 from the bile canalicular (apical) membrane to the sinusoidal (basolateral) membrane in a human hepatoma cell line as well as in rat liver accompanied by cholestasis (Kubitz et al., 2001).

Indeed, Abcc2 is known to be phosphorylated by PKCα (one of the conventional PKC (cPKC) family) and PKCe (nPKC) in vitro (Ito et al., 2005; Wimmer et al., 2008). In spite of growing evidence showing the contribution of PKCs to the post-translational regulation of Abcc2 in hepatocytes, little is known about whether it holds true for other tissues.

Several reports have also shown an influence of PKC on ABCB1. ABCB1 mRNA expression is increased by PKCs activator phorbol ester, and in some pathological model (Chaudhary and Roninson, 1992; Kameyama et al., 2008). Moreover, ABCB1 protein itself is also a phosphorylation substrate of PKCα, although its functional significance has not been well established (Ahmad et al., 1994; Goodfellow et al., 1996). These studies are mostly performed using certain in vitro cell culture models where
signaling molecules are heterogeneously expressed and likely different from each other. Role of PKCs on the expression / function of ABCB1 should be better evaluated in a model reflecting in vivo situation.

In this study, we tried to compare the effect of cPKC activation on the expression and function of Abcc2 and Abcb1 using in situ rat small intestine. Our results indicated that cPKC activation down-regulated Abcc2 function as a result of its reduced protein expression in BBM, which correlates with a reduced interaction with ezrin, a dominant ERM protein expressed in the small intestine. Such effect was selective because Abcb1 expression and function were minimally affected by the same treatment.
Materials and Methods

Chemicals and antibodies

Thymeleatoxin (Thx); acivicin; and rabbit anti-PKCα, δ, and ε antibodies were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). Gö6976 was obtained from Calbiochem (La Jolla, CA). The fluorescein labeled goat anti-rabbit IgG used in the immunofluorescence analysis and monochlorobimane (MCB) were from Molecular Probes (Eugene, OR). Rabbit anti-ERM and C-terminally phosphorylated ezrin (Thr567)/radixin (Thr564)/moesin (Thr558) (p-ERM) antibodies were purchased from Chemicon (Temecula, CA). The horseradish peroxidase-linked secondary antibodies used in the immunoblot analysis and mouse anti-ezrin monoclonal antibody (4A5) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Abcc2 polyclonal antibody was raised against the 12-amino acid sequence at the carboxyl terminus of rat Abcc2. Protein A agarose beads were purchased from Pierce Chemical Company (Rockford, IL). All other chemicals and solvents were of an analytical grade.

Animals

Male Sprague-Dawley (SD) rats and Eisai hyperbilirubinemic rats (EHBR), 200-300g in weight, were used throughout the experiments (SLC Japan Inc., Tokyo, Japan). All animals were treated humanely in accordance with the guidelines issued by the National Institutes of Health.

Everted sacs studies

The everted sacs experiment was performed as described elsewhere (Hakata et al., 2005). The rats were anesthetized with diethyl ether and sacrificed by exsanguination from the abdominal aorta. Then, the jejunum was immediately removed and rinsed in
ice-cold saline. A 5 cm segment of the jejunum was isolated for use in the everted sac studies. The intestinal segment was slid onto a piston and the epithelial surface was exposed. The segment was ligated to syringes at both ends and pretreated with modified Krebs-Ringer bicarbonate buffer (mKRBB: 5 mM KCl; 120 mM NaCl; 15 mM HEPES, pH 7.4; 24 mM NaHCO₃; 1 mM MgCl₂; 2 mM CaCl₂), pH 6.0, containing 40 mM glucose and 1 mM acivicin to inhibit γ-glutamyltransferase with or without Thx for 20 min. In some experiments, sacs were incubated with 200 nM Gö6976 at the same time. The solution was gassed with O₂/CO₂ (95:5) at 37°C. Then a final concentration of 20 μM MCB, a precursor of glutathione S-bimane (GS-B), was added to both sides. Aliquots from inside the sac and from outside the sac were collected at 10, 20, 30, and 40 min. Finally the sac was washed with ice-cold saline and the intestinal mucosa was scraped with a glass slide and homogenized to allow measurement of the intramucosal contents. All specimens were diluted with EDTA (0.1% w/v): metaphosphoric acid (25% w/v), 7:2, and were then subjected to reverse phase-high performance liquid chromatography with spectrofluorometric detection at wavelengths of 386 nm (excitation) and 476 nm (emission) to quantify GS-B.

To investigate Abcb1-mediated efflux, a 5 cm segment of the ileum was isolated. The everted sac studies were carried out under the same conditions as those for GS-B transport except 1 mM acivicin was not used, and 5 μM rhodamine123 (Rho123), an Abcb1 substrate, was added to the donor side. Samples were obtained from the receiver side every 10 min for 40 min. These samples were subjected to fluorescence analysis at wavelengths of 505 nm (excitation) and 535 nm (emission) with a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan).

Intestinal brush border membrane (BBM) preparation
A BBM fraction from the rat small intestine was prepared using calcium precipitation methods (Kessler et al., 1978; Li et al., 2003) with some modifications. The intestinal mucosa was scraped off gently with a glass slide and homogenized in 10 ml/g wet weight of tissue, 50 mM mannitol, 2 mM Tris/HCl (pH 7.5), 1 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 μg/ml aprotinin at 1,800 rpm for 5 min with an Acehomogenizer AM-1 (Nihonseiki Kaisha Ltd., Tokyo, Japan). The suspension was supplemented with 10 mM CaCl2 and incubated for 15 min at 4°C, before being centrifuged at 3,000 g for 20 min. The resulting supernatant was collected and centrifuged at 27,000 g for 30 min. The pellets were resuspended in 50 mM mannitol and 10 mM HEPES/Tris (pH 7.5) and were centrifuged at 27,000 g for 30 min. The resulting pellet was resuspended in 300 mM mannitol, 10 mM HEPES/Tris (pH 7.5), 1 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 μg/ml aprotinin with a syringe and a 26-gauge needle and was stored at -80°C until use. The protein concentration was determined by the Lowry method using bovine serum albumin (BSA) as a standard.

**SDS-PAGE and immunoblot analysis**

The homogenate and BBM from the rat intestine were loaded onto an 8.5% polyacrylamide slab gel containing 0.1% SDS and then transferred to an Immobilon-P Transfer Membrane filter (Millipore Co., Bedford, MA) by electrophoresis. The membrane was blocked for 1 h at room temperature or overnight at 4°C with TTBS (Tris-buffered saline with 0.05% Tween 20) containing 3% BSA and was probed at room temperature for 1 h or at 4°C overnight with primary antibodies diluted with TTBS containing 0.1% BSA. The membrane was incubated for 1 h at room temperature with secondary antibodies conjugated with horseradish peroxidase and diluted with TTBS containing 0.1% BSA. The membrane was detected by LAS1000 (Fuji photo film Co.)
Immunofluorescence analysis

To detect PKCα, small tissue blocks embedded in Tissue-Tek O.C.T. Compound (Sakura Finetechical Co. Ltd., Tokyo, Japan) were snap frozen in liquid nitrogen. The frozen block was then used to prepare 6 μm thick sections at -25°C, which were then fixed in acetone at room temperature for 10 min. The sections on the slides were hydrated in PBS for 15 min twice and blocked for 1 h with 3% BSA, and then anti-PKCα antibodies (1:50) diluted in PBS were applied for 1 h. After rinsing with PBS, the slides were incubated for 1 h with FITC goat anti-rabbit IgG (1:250) diluted in PBS. The samples were mounted in VECTASHIELD with propidium iodide (Vector Laboratories, Burlingame, CA) for counterstaining of nuclei. To detect C-terminally phosphorylated ezrin (p-ERM), the tissue blocks were subjected to trichloroacetic acid (TCA) fixation as described previously (Hayashi et al., 1999). Briefly, the tissue blocks were immersed in ice-cold 10% TCA for 1 h, before being washed with PBS containing 30 mM glycine (G-PBS). Then tissue sections were prepared from the tissue blocks fixed with TCA under the same operating conditions to detect PKCα. The TCA-fixed sections were permeabilized for 15 min with 0.2% Triton X-100 in G-PBS and washed with G-PBS three times. The sections were blocked for 1 h with 1% BSA in G-PBS, and then anti-p-ERM antibodies (1:50) diluted in G-PBS were applied overnight at 4°C. After rinsing with PBS, the slides were incubated for 1 h with fluorescein labeled goat anti-rabbit IgG (1:100) diluted in G-PBS. The samples were analyzed using a confocal laser scanning microscope, LSM510 type (Carl Zeiss, Jena, Germany).

Immunoprecipitation assay
The following procedures were performed at 4°C. The intestinal mucosa was scraped and snap frozen in liquid nitrogen before being stored at -80°C until use. The mucosa was homogenized with twenty vertical strokes of a Teflon homogenizer in 10 ml/g tissue wet weight of lysis buffer containing 20 mM NaPi (pH 7.4), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 60 mM n-octyl-β-D-glucopyranosid, 1% (v/v) Triton X-100, and protease inhibitor (1 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 5 μg/ml aprotinin) and was lysed for 30 min. The tissue homogenate was centrifuged at 20,000 g for 30 min, and then the resulting supernatant was collected as tissue lysate and rotated for 1 h with PANSORBIN cells (Calbiochem Inc., Darmstadt, Germany). The lysate was centrifuged at 20,000 g for 30 min. The supernatant was rotated for 1 h or overnight with anti-ezrin monoclonal antibody. Then Protein A agarose beads were added to the lysate and rotated for 1 h. The beads were sedimented at 20,000 g for 1 min and washed three times with lysis buffer. Finally, elution buffer containing 10 mM Tris/HCl (pH 6.5), 3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 8 M urea, and 0.001% bromophenol blue was added and boiled at 95°C for 5 min. The suspension was centrifuged, and then the supernatant was subjected to SDS-PAGE followed by immunoblot analysis.

Statistical analysis

All data are represented as mean ± S.E. Data were analyzed statistically using the two-tailed Student’s t-test or Bonferroni’s correction for multiple comparisons when appropriate. Differences between means at the level of p<0.05 were considered to be significant.
Results

The effect of thymeleatoxin (Thx) on the distribution of PKC isoforms in the rat intestine

Thx, an analogue of mezerein, is known as a selective activator of the cPKC family, which includes the PKCα, PKCβI, PKCβII, and PKCγ isoforms. Since activated PKCs rapidly change their subcellular localization, especially from the soluble cytosolic fraction to the membranous fraction (Newton, 1995), the localization of PKC was analyzed after Thx treatment. We selected PKCα as a readout of cPKC activation. Immunoblot analysis showed that PKCα was translocated to the membranous fraction when treated with Thx, while PKCδ and PKCε, both of which are nPKCs, were not (Fig. 1A). Immunofluorescence analysis also demonstrated that PKCα accumulated near to the apical membrane of intestinal epithelial cells after treatment with Thx (Fig. 1B). These results indicate that Thx selectively stimulate cPKC in rat small intestine.

The effect of Thx on GS-B and rhodamin123 transport

We next tried to monitor the functional changes in Abcc2 and Abcb1 after treatment with Thx.

MCB is passively taken up by the cells to be conjugated with GSH to produce GS-B, which is a fluorescent substrate of Abcc2 (Oude Elferink et al., 1993). The efflux of GS-B to the mucosal side of the jejunum was monitored to evaluate the effect of Thx on the function of Abcc2 in an everted sacs experiment since it is located on mucosal side of the intestinal epithelia. Time courses of GS-B efflux to the mucosal and serosal sides in everted sacs are shown in Fig. 2A and 2B, respectively. Thx-treatment significantly reduced the cumulative GS-B efflux for 40 min to the mucosal side from 30.3 ± 2.1 nmol/cm (control) to 18.1 ± 1.6 nmol/cm (Thx) (Fig. 2A), without affecting GS-B efflux.
to the serosal side (Fig. 2B) or intramucosal GS-B accumulation (Fig. 2C). Moreover, Gö6976, a cPKC specific inhibitor, blocked the inhibitory effect of Thx on GS-B efflux (Fig. 2A). Although Thx reduced the production of GS-B (intramucosal + transport to mucosal side + transport to serosal side) from 62.4 ± 2.7 nmol/cm (control) to 45.3 ± 4.3 nmol/cm (Thx) (p=0.027), the decrease was most prominently observed for the transport to mucosal side, where Abcc2 is located. To further confirm the dominant contribution of Abcc2 on the efflux of GS-B in rat small intestine, the same experiments were performed in Eisai hyperbilirubinemnic rat (EHBR), whose Abcc2 expression is hereditarily defective. As a result, GS-B efflux into mucosal side of EHBR intestine was decreased to about 40% of that in control SD rat intestine, while GS-B efflux into serosal side was not different each other. Moreover, Thx had no effect on GS-B transport into both mucosal and serosal sides in EHBR intestine (Fig. 2D, E). These results indicate that Abcc2 is functionally down regulated by Thx treatment in rat small intestine.

As a comparative experiment, the transport of Rho123 across the intestinal sacs in both absorptive (mucosal to serosal) and secretory (serosal to mucosal) directions was assessed (Fig. 3A). Abcb1 is located on the mucosal side of the intestine, thus vectorial transport of Rho123 (Abcb1 substrate) in the serosal to mucosal direction is readily observed (Fig. 3A). Although, Thx treatment slightly increased Abcb1 mediated Rho123 transport from the serosal to the mucosal side, the differences in secretory flux, absorptive flux, and intramucosal accumulation of Abcb1 (Fig. 3B) were not statistically significant between the control and Thx-treated groups.

**The effect of Thx on the apical localization of Abcc2 and Abcb1 in the intestinal brush border membrane**
The expression of Abcc2 in the total homogenates and BBM prepared from Thx treated jejunum segments were examined by immunoblot analysis. Alkaline phosphatase activity was measured in the homogenate and BBM to calculate the enrichment of the BBM. The ratio was similar between the control and Thx treated segments (approximately 10-fold, data not shown), which was consistent with previous reports (Kessler et al., 1978). The expression of Abcc2 in the BBM fraction was reduced to approximately 50% of that of the control by Thx treatment for 20min, while that in the homogenate remained unchanged (Fig. 4A). On the other hand, the expression of Abcb1 in both homogenate and BBM was not changed by the Thx treatment (Fig. 4B).

**Distribution of C-terminally phosphorylated ezrin in the rat intestinal brush border membrane**

Abcc2 is physically supported by its interaction with the ERM protein family as demonstrated in hepatocytes and intestinal cell lines (Kikuchi et al., 2002; Wang et al., 2006; Yang et al., 2007). Without the presence of such scaffold proteins, Abcc2 on the apical membrane surface is selectively reduced. These ERM proteins are phosphorylated by a specific threonine residue located near the C-terminal region. This reaction produces the active form of ERM proteins (Matsui et al., 1998). Based on these previous findings, we hypothesized that membrane surface Abcc2 is reduced as a result of reduced ERM expression and/or function. As the predominant ERM protein in the small intestine is ezrin (Hayashi et al., 1999), we quantified the expression of ezrin and its phosphorylated form (p-ezrin) after Thx treatment. Although the amount of total ezrin was constant irrespective of the treatment (Fig. 5A), p-ezrin was reduced to about 60% of that of the control by the Thx treatment. This effect is certainly mediated by the
activation of cPKC because the amount of p-ezrin was recovered to the control level by
the concomitant use of Gö6976 (Fig. 5A, B). Immunofluorescence analysis indicated a
strong signal from p-ezrin near to the BBM region in the control tissue. Thx treatment
did not affect the distribution of p-ezrin but attenuated its signal intensity (Fig. 5C).

**The effect of Thx on the protein-protein interactions of Abcc2 and Abcb1 with ezrin**

To get an insight into the molecular mechanism involved in the down-regulation of
Abcc2 surface expression by cPKC activation, we tried to investigate the molecular
interaction of Abcc2 with ezrin. In control tissues without any treatment, Abcc2 was
readily co-immunoprecipitated with ezrin as reported previously (Fig. 6) (Kikuchi et al.,
2002; Yang et al., 2007). The interaction between ezrin and Abcc2 was specific since
Abcc2 was not detected when pre-immune mouse IgG was used for the
immunoprecipitation (data not shown). The total expression of ezrin (Fig. 6, Input) and
the amount of ezrin immunoprecipitated with anti-ezrin monoclonal antibody were not
changed by Thx treatment for 20min, while the amount of p-ezrin in the
immunoprecipitate was decreased by Thx treatment, but this decrease could be reversed
by the concomitant use of Gö6976 (Fig. 6, IP). Moreover, the amount of Abcc2
cooprecipitated with ezrin decreased in parallel with p-ezrin after Thx treatment,
and the concomitant use of Gö6976 reversed the decrease in the amount of Abcc2
cooprecipitated with ezrin (Fig. 6, IP). Although, the interaction of Abcb1 and
ezrin was also confirmed, the amount of Abcb1 in the immunoprecipitate was not
decreased by the Thx treatment (Fig. 6, IP).
**Discussion**

Multiple transporters undergo post-translational regulation including exocytic insertion or endocytic retrieval from the plasma membrane surface. Intestinal glucose transporter 2 (Kellett, 2001), cystic fibrosis transmembrane conductance regulator (Prince et al., 1994), and sodium/hydrogen exchanger isoform 3 (Janecki et al., 1998) are well characterized examples, and some of these are under the control of PKC dependent signaling pathways (Janecki et al., 1998; Kellett, 2001). And also, we have previously demonstrated that nPKC activation is involved in the rapid internalization of Abcc2 in rat hepatocytes under oxidative stress condition (Sekine et al., 2006).

PKC isoforms can be classified into Ca\(^{2+}\)-dependent cPKCs including PKC\(\alpha\), \(\beta I\), \(\beta II\), and \(\gamma\); Ca\(^{2+}\)-independent nPKCs including PKC\(\delta\), \(\varepsilon\), \(\theta\), \(\eta\), and \(\mu\); and atypical PKCs including PKC\(\lambda\), \(\iota\), and \(\zeta\) (Hofmann, 2004). At least 5 isoforms including PKC\(\alpha\), \(\beta II\), \(\delta\), \(\varepsilon\), and \(\zeta\) are detected in rat intestinal epithelial cells (Saxon et al., 1994). In this study, we first focused on cPKC to determine its role in the regulation of the intestinal ABC transporters Abcc2 and Abcb1. As a result, we were able to demonstrate that a short exposure to Thx (20 min) reduced the transport activity of Abcc2 without changing the total protein expression (Figs. 2A, 4A). There was a clear parallel between the protein content of Abcc2 and the transport activity, suggesting that cPKC activation induces a decrease in the transport activity of Abcc2 as a result of decreased protein content of Abcc2 on BBM surface. It is reported that the half-life of Abcc2 in the canalicular membrane is as long as 27 h (Jones et al., 2005). If Abcc2 in the BBM fraction is as stable as that in the bile canalicular membrane, the decrease in BBM observed without a change in the total amount of Abcc2 is probably due to accelerated internalization rather than impaired trafficking of newly synthesized molecules to the BBM. This is the first demonstration that Abcc2 is rapidly internalized in the intestine in a cPKC-dependent manner.
manner. Importantly, this effect is selective for Abcc2 because Abcb1 expression and function are not decreased under the same experimental conditions (Figs. 3, 4B), suggesting differential mechanisms governing the post-translational regulation of these two intestinal ABC transporters.

The membrane surface localization of Abcc2 is physically supported by ERM proteins, which act as molecular anchors connecting Abcc2 to F-actin cytoskeleton. Radixin performs this task in hepatocytes (Kikuchi et al., 2002), and both radixin and ezrin in the intestinal Caco2 cell line (Yang et al., 2007). We hypothesized that the reduced Abcc2 expression in the BBM is caused by the reduced interaction of Abcc2 with ezrin, the predominant ERM isoform expressed in intestinal epithelia (Hayashi et al., 1999). Our immunoblot results showed that the total amount of ezrin in the cell lysate was unchanged, but the amount of p-ezrin (T567 phosphorylated ezrin) in the BBM fraction was reduced to approximately 60% of that of control by Thx treatment (Figs. 5A, 5B). The phosphorylation of T567, which is located near the C-terminal of ezrin, is related to the activation of ezrin from its dormant closed form to its active open form. The active open form can efficiently bind to membrane (proteins) with its N-half domain and also to F-actin with its C-half domain. Our immunoprecipitation studies also indicated decreased p-ezrin in immunoprecipitated ezrin. Although the amount of p-ezrin recovered from cell lysate was thought to be small because of its tight binding to the cytoskeletal F-actin, we confirmed the reproducibility of dephosphorylation of ezrin by Thx treatment in this experimental model. Furthermore, the association of Abcc2 with ezrin was confirmed in the rat small intestine, and which was reduced by Thx treatment (Fig. 6). Supporting the importance of p-ezrin as an active molecule associated with Abcc2, the extent of the reduction of Abcc2 in the BBM paralleled that of p-ezrin after Thx treatment. It is worth noting that the concomitant use of Gö6976
completely cancelled out the effect of Thx on Abcc2 as well as its effect on p-ezrin, while Gö6976 alone did not affect the expression of either of these two proteins. These results indicate that the expression of Abcc2 in the BBM does not require cPKC activity under basal conditions, while the decrease of p-ezrin and redistribution of Abcc2 after Thx treatment is indeed mediated by the activation of cPKC.

Multiple kinases (Matsui et al., 1998; Wald et al., 2008) and putative protein phosphatase(s) (Zhu et al., 2007) are involved in the phosphorylation and dephosphorylation of the C-terminal threonine of ERM proteins, in order to regulate the balance between their active and inactive forms. PKCα classified into cPKC is one of the kinases that directly associates with ezrin to phosphorylate its C-terminus threonine (T567) as demonstrated in 2C4 fibrosarcoma cells (Ng et al., 2001). However, our results showed that p-ezrin rather decreased when cPKC was stimulated by Thx. One of the possible explanations for the discrepancy is that PKCα is not a major kinase involved in the phosphorylation of ezrin T567 at least under the conditions of our rat small intestine model, but other protein kinases or phosphatases directly involved in the modification of ezrin T567 may be phosphorylated by cPKC so as to be inactivated or activated. This possibility needs to be investigated in the future study.

It is reported that the association of ABCB1 with cytoskeletal actin through ERM proteins plays a key role in the cellular localization and function of ABCB1 in human cells of lymphoid origin (Luciani et al., 2002). Our immunoprecipitation studies also confirmed the interaction of Abcb1 with ezrin in rat small intestine (Fig. 6). However, the amount of Abcb1 co-immunoprecipitated with ezrin was not reduced by Thx treatment, in spite of a decrease in p-ezrin. One of the possible explanations for the differential sensitivity of Abcb1 and Abcc2 is that Abcb1 can associate with the phosphorylated (p-ezrin) as well as the dephosphorylated form of ezrin, while Abcc2 only
associates with p-ezrin. Although phosphorylation of the C-terminal threonine is believed to open up the NH2 terminal-to-COOH terminal (N-C) binding of ezrin and to transform ezrin into its active form with accessible domains for binding to membrane proteins and F-actin, dephosphorylated dormant ezrin does have an NH2 terminal membrane binding site (Zhu et al., 2007). Indeed, only 20% of ezrin is phosphorylated at its C-terminal (T567) (Zhu et al., 2007), while all ezrin molecules (both phosphorylated and unphosphorylated forms) are associated with the apical membrane fraction of gastric parietal cells (Hanzel et al., 1991). In our experimental model, the content of total ezrin in the BBM fraction was not changed in spite of decrease in p-ezrin (Fig. 5A, B). Based on these considerations, it is possible that ezrin can bind to Abcb1 irrespective of its phosphorylation status. The molecular mechanism of interaction between ERM and Abcb1 need to be further evaluated.

Variable effects were reported in ERM knockout/down experimental models. Kikuchi et al. reported a selective reduction of Abcc2 from the canalicular surface of radixin knockout mice hepatocytes, while the effect on Abcb1 was apparently minimal (Kikuchi et al., 2002). On the other hand, expression of ABCB1/Abcb1 as well as of other ABC transporters in the apical membrane was similarly disrupted in other cases including Caco2 cells stably suppressed with radixin or ezrin (Yang et al., 2007), primary cultured rat hepatocytes transiently suppressed with radixin (Wang et al., 2006), and in the liver of patients with chronic cholestatic liver disease, where radixin expression is compromised (Kojima et al., 2003). Since radixin and ezrin are also important for the formation of apical microvilli structures (Saotome et al., 2004; Wang et al., 2006), the latter global effects might be partly explained by the disruption of apical membrane structure itself. Considering the short exposure period to Thx (20 min) and selective loss of Abcc2 from the membrane in our study, dissociation of Abcc2 from membrane
cytoskeleton F-actin as a result of p-ezrin down-regulation likely precedes such global disruption of apical membrane structure.

In conclusion, cPKC activation induced a selective reduction of Abcc2 function by decreasing its membrane surface expression in the rat intestine. There was a clear correlation between apical localization of Abcc2 and phosphorylation status of ezrin, which links Abcc2 to membrane cytoskeleton F-actin. This is the first demonstration of the post-translational regulation of Abcc2 by PKC in the small intestine.
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Tauroursodeoxycholic acid exerts anticholestatic effects by a cooperative cPKC{alpha}-/PKA-dependent mechanism in rat liver. *Gut*.


Figure Legends

Fig. 1. Effect of Thx treatment on the distribution of PKC isoforms (α, ε, and δ) in the rat jejunum. (A) Immunoblot analysis of PKC subtypes in the BBM fraction prepared from rat jejunum. Everted sac segments from the jejunum were incubated with or without (control) 50 nM Thx, subsequently followed by preparation of the BBM fraction and frozen sections. The BBM (10 μg protein) was subjected to immunoblot analysis and detected with specific antibodies for PKCα, ε, and δ (upper). Band densities were quantified (lower). Each value represents the mean ± S.E. (n= 3). *p<0.05 compared with the control group. (B) Immunofluorescence and confocal laser scanning microscopy of rat intestinal villi. Frozen sections (6 μm in thickness) were stained with anti-PKCα antibody (green) and propidium iodide for nuclei (red).

Fig. 2. Time profiles for GS-B efflux into (A), (D) the mucosal side, (B), (E) the serosal side and (C) intramucosal GS-B accumulation at 40 min. Everted sac segments of the jejunum isolated from (A), (B), (C) SD rats and (D), (E) EHBR were used to examine the transport of GS-B. The everted sacs were filled with mKRBB and incubated without (●, ▲) or with 50 nM Thx (○, △), 0.2 μM Gö6976 (◆), or 50 nM Thx and 0.2 μM Gö6976 (□) for 20 min. MCB was added to both the mucosal and serosal sides (20 μM). Each value represents the mean ± S.E. (n = 3 for the control, Thx, Gö6976 and Thx + Gö6976 groups in SD rats, 4 for the EHBR groups). **p<0.01 compared with the control group. †p<0.05 compared with the Thx treatment group.

Fig. 3. Time profiles for Rho123 transport across the intestinal epithelia. (A) Transport from the serosal to the mucosal side (○, ■) or from the mucosal to the serosal side (▲) was examined. Everted sac segments from the ileum were used to examine the
transport of Rho123. The everted sacs were filled with mKRBB and incubated without or with 50 nM Thx for 20 min. Rho123 was added to the donor side (5 μM). Serosal to mucosal transport of Rho123 was examined in the presence (●) or absence (○) of Thx. (B) At the end of the experiment (40 min), intramucosal accumulation of Rho123 was examined. Each value represents the mean ± S.E. (n= 4).

Fig. 4. Effect of Thx treatment on the expression levels of (A) Abcc2 and (B) Abcb1 in the homogenate and BBM fraction prepared from rat jejunum. Everted sac segments from the jejunum were filled with mKRBB and incubated for 20 min without (control, open column) or with 50 nM Thx (closed column). Then mucosae were homogenized and separated into BBM fractions. Twenty μg proteins of homogenate and BBM were subjected to immunoblot analysis and detected with anti-Abcc2 polyclonal antibody and with anti-Abcb1 monoclonal antibody (C219). Band densities of Abcc2 and Abcb1 were quantified. Each value represents the mean ± S.E. (n= 3). *p<0.05 compared with the control group.

Fig. 5. Effect of Thx treatment on the expression levels and distribution of p-ezrin (phosphorylation at Thr567) in the rat jejunum. (A) Immunoblot analysis of ezrin and p-ezrin in the BBM in the presence or absence of Thx and/or Gö6976. Everted sacs from the jejunum were filled with mKRBB and incubated without or with 50 nM Thx, 0.2 μM Gö6976, or 50 nM Thx and 0.2 μM Gö6976 for 20 min. Then mucosae were homogenized and separated into BBM fractions. Twenty μg protein of BBM was subjected to immunoblot analysis using anti-ERM and anti-p-ERM antibodies. Data are representative of at least three independent experiments. (B) The relative band densities of p-ezrin were quantified. Each value represents the mean ± S.E. (control and Thx
groups (n=6), Gö6976 and Thx + Gö6976 groups (n=3)).  (C) Immunofluorescence and confocal laser scanning microscopy of rat intestinal villi.  Everted sacs from the jejunum were incubated without (control) or with 50 nM Thx for 20 min.  Frozen sections (6 μm in thickness) of TCA-fixed tissue were stained with anti-p-ERM antibody and examined with a laser confocal microscope.  The results are displayed raw confocal images and 3D fluorescence intensity plots.  Scale bars: 50 μm.

Fig. 6.  Effect of Thx treatment on the protein-protein interaction between Abcc2 and ezrin.  Everted sacs were treated with Thx and/or Gö6976 as in Fig. 5, homogenized, and lysed for 30 min.  Then, the lysates were subjected to immunoprecipitation (IP) using anti-ezrin monoclonal antibody.  The lysates (Input) and immunoprecipitates were subjected to immunoblot (IB) analysis using anti-ERM, p-ERM, Abcc2, and Abcb1 antibodies.  Band densities were quantified and the mean density of control was set at 100%.  Each value represents the mean ± S.E. (n=3 except for the Thx + Gö6976 group of Abcb1 (n=2)).
**Fig. 2**

**A**

GS-B efflux to mucosal side (nmol/cm) vs. Time (min).

- **Control**
- **Thx**
- **Gö6976**
- **Thx+Gö6976**

**B**

GS-B efflux to serosal side (nmol/cm) vs. Time (min).

- **Control**
- **Thx**
- **Gö6976**
- **Thx+Gö6976**

**C**

Content of intramucosal GS-B (nmol/cm).

- Thx: -
- Gö6976: -
- Thx: +
- Gö6976: +

**D**

GS-B efflux to mucosal side (nmol/cm) vs. Time (min).

- **SD rat (Control)**
- **SD rat + Thx**
- **EHBR**
- **EHBR + Thx**

**E**

GS-B efflux to serosal side (nmol/cm) vs. Time (min).

- **SD rat (Control)**
- **SD rat + Thx**
- **EHBR**
- **EHBR + Thx**
Fig. 3

A

Rho123 transport (pmol/cm)

- O S to M
- S to M + Thx
- ▲ M to S

Time (min)

B

Content of intramucosal Rho123 (pmol/cm)

(×10²)

Thx - +
Fig. 4

A

Control Thx
Abcc2

Density (% of Control)

0 20 40 60 80 100 120 140

Homogenate BBM

B

Control Thx
Abcb1

Density (% of Control)

0 20 40 60 80 100 120 140

Homogenate BBM

*
**Fig. 5**

A. 

B. 

C. 

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Fig. 6

IB: ezrin

(pT567)

IP: ezrin

Abcc2

Abcb1

(%) of Control

X±S.E. 100 93±8 94±11 94±14

X±S.E. 100 103±12 103±9 99±12

X±S.E. 100 59±13 122±29 80±19

X±S.E. 100 67±11 122±17 104±9

X±S.E. 100 114±22 122±25 125

Thx – + – +

Gö6976 – – + +