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MOLECULAR TOXICOLOGY

Acute regulation of multidrug resistance-associated protein 2 localization and activity by cAMP and estradiol-17β-D-glucuronide in rat intestine and Caco-2 cells

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Abstract Multidrug resistance-associated protein 2 (MRP2) is an ATP-dependent transporter expressed at the brush border membrane of the enterocyte that confers protection against absorption of toxicants from foods or bile. Acute, short-term regulation of intestinal MRP2 activity involving changes in its apical membrane localization was poorly explored. We evaluated the effects of dibutyryl-cAMP (db-cAMP), a permeable analog of cAMP, and estradiol-17β-D-glucuronide ($E_2$17G), an endogenous derivative of estradiol, on MRP2 localization and activity using isolated rat intestinal sacs and Caco-2 cells, a model of human intestinal epithelium. Changes in MRP2 localization were studied by Western blotting of plasma membrane (PM) vs. intracellular membrane (IM) fractions in both experimental models, and additionally, by confocal microscopy in Caco-2 cells. After 30 min of exposure, db-cAMP-stimulated sorting of MRP2 from IM to PM both in rat jejunum and Caco-2 cells at 10 and 100 μM concentrations, respectively, with increased excretion of the model substrate 2,4-dinitrophenyl-*S*-glutathione. In contrast, $E_2$17G (400 μM) induced internalization of MRP2 together with impairment of transport activity. Confocal microscopy analysis performed in Caco-2 cells confirmed Western blot results. In the particular case of $E_2$17G, MRP2 exhibited an unusual pattern of staining compatible with endocytic vesiculation. Use of selective inhibitors demonstrated the participation of cAMP-dependent protein kinase and classic calcium-dependent protein kinase C in db-cAMP and $E_2$17G effects, respectively. We conclude that localization of MRP2 in intestine may be subjected to a dynamic equilibrium between plasma membrane and intracellular domains, thus allowing for rapid regulation of MRP2 function.

Keywords MRP2 · Intestine · cAMP · $E_2$17G · Brush border membrane · Food toxicants

Introduction

Multidrug resistance-associated protein 2 (MRP2) belongs to the superfamily of ATP-Binding Cassette (ABC) transporters, and is involved in extrusion of a wide variety of endogenous metabolites and xenobiotics, mostly conjugated with glucuronic acid, glutathione and sulfate (Keppler and König 1997). It is mainly expressed in epithelia of organs with high secretory activity like liver, kidneys and intestine (Keppler and König 1997; Gotoh et al. 2000; Mottino et al. 2000; Van Aubel et al. 2000). In rat small intestine, Mrp2 is localized to the apical, brush border membrane (BBM) of the mature enterocyte and is maximally expressed at the tip of the villus (Mottino et al. 2000). It follows an oral to caudal gradient along the small intestine with maximal expression in jejunum, decreasing towards the ileum (Mottino et al. 2000). Significant expression of MRP2 was also detected in human duodenum (Fromm et al. 2000). This particular distribution confers MRP2 a toxicologically relevant function in preventing absorption of drugs and food additives and contaminants, as was specifically demonstrated for the abundant food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Dietrich et al. 2001). Such distribution also indicates a function in

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prevention from reabsorption of biliary constituents, including conjugates of endogenous metabolites, drugs and contaminants (Tocchetti et al. 2016).

Localization of MRP2 at the apical membrane of epithelial cells is dependent on the presence of the ezrin–radixin–moesin (ERM) family of proteins that links the transporter to integral membrane proteins; this was initially demonstrated for radixin in hepatocytes (Kikuchi et al. 2002). Distribution of hepatic MRP2 between the plasma membrane and the intracellular subapical reservoir is subjected to a dynamic equilibrium (Crocenzi et al. 2012). It was demonstrated in human liver cell models and in rat liver that cholestatic agents induce internalization of MRP2 and other canalicular transporters from the plasma membrane to a subapical compartment, with consequent loss of activity (Kubitz et al. 2001; Mottino et al. 2002; Crocenzi et al. 2003; Saeki et al. 2011; Schonhoff et al. 2013). Such alterations in MRP2 localization involve participation of different intracellular messengers, mainly protein kinases, and occur in an acute and reversible manner. Activation of classic calcium-dependent protein kinases C (cPKCs) were particularly involved in experimental models of cholestasis such as those induced by estradiol-17β-D-glucuronide ($E_2$17G) (Crocenzi et al. 2008), oxidative stress (Sekine et al. 2008) and phorbol-12-myristate-13-acetate (PMA) (Kubitz et al. 2001). In contrast, cyclic adenosine monophosphate (cAMP) prevents from Mrp2 endocytosis induced by $E_2$17G (Mottino et al. 2002), taurolithocholate (Miszczuk et al. 2015) and by oxidative stress, the latter one mediated by cAMP-dependent protein kinase (PKA) (Sekine et al. 2008).

The regulation of MRP2 localization in small intestine was much less studied. An interaction between Mrp2 and ezrin, the member of the ERM family protein that is dominantly expressed in the intestine, was demonstrated in rats by Nakano et al. (2009). These same authors found that selective activation of cPKCs with thymeleatoxin decreased the interaction between ezrin and Mrp2 with consequent loss of Mrp2 from the BBM. Whether a dynamic interchange of MRP2 between the BBM and a subapical compartment exists in the intestine has not been demonstrated. The impact of agents that promote internalization or insertion of MRP2 from/to the canalicular membrane, such as $E_2$17G and cAMP, on this dynamic balance in intestine has neither been tested. These agents could help understand if intestinal MRP2 is subjected to post-translational regulation as demonstrated in liver and whether transport activity is conditioned by such regulation.

In the current study, we assessed the effect of cAMP and $E_2$17G on MRP2 localization in two experimental models, rat intestinal sacs and Caco-2 cells, a model of human intestinal epithelium. The study may be not only of toxicological but also of physiological and pathophysiological relevance since (1) cAMP mediates the effect of intestinal hormones acting locally such as glucagon-like peptide 2 (GLP-2) (Villanueva et al. 2010) and (2) cholestatic estrogen derivatives such as $E_2$17G are expected to increase significantly in bile during pregnancy, estrogen replacement therapy or contraception treatments (Vore et al. 1997). We additionally evaluated the participation of PKA and cPKCs in cAMP and $E_2$17G effects.

Materials and methods

Chemicals

Bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), dibutyryl cyclic-AMP sodium salt (db-cAMP), estradiol-17β-D-glucuronide ($E_2$17G), Gö6976, leupeptin, MK571, pepstatin A and phenylmethylsulfonyl fluoride (PMSF) were from Sigma-Aldrich. KT5720 was from Santa Cruz Biotechnology. DMSO was from Merck. All other chemicals were of analytical grade purity. The glutathione-conjugated derivative of CDNB, 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), was synthesized with the use of 1-fluoro-2,4-dinitrobenzene and glutathione as described by Sokolovsky et al. (1964).

Experiments in isolated rat intestinal sacs

Animals and sample collection

Adult male Wistar rats (310–330 g) were provided by Centro de Medicina Comparada (UNL-CONICET, Esperanza, Argentina). They were maintained on a standard diet and water ad libitum, and housed in a temperature- and humidity-controlled room under a constant 12-h light, 12-h dark cycle. The animals were fasted for 12 h before the experiments. At the time of the experiments they were anesthetized (ketamine: 100 mg/kg b.w.; xylazine: 15 mg/kg b.w.; i.p.) and exsanguinated by cardiac puncture. The proximal jejunum (about 32-cm length) was removed, carefully rinsed with ice-cold saline and divided into eight segments (about 4-cm length). While non-everted sacs were used in determination of Mrp2 localization by membrane fractionation followed by Western blotting (Sect. 2.2.2), everted sacs were used in transport activity experiments (Sect. 2.2.3).

All procedures involving animals were performed as outlined in the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.

Assessment of Mrp2 distribution between brush border (BBM) and intracellular (IM) membrane fractions

The effects of db-cAMP and $E_2$17G were tested alone or in combination with the PKA inhibitor KT5720 or the cPKC inhibitor Gö6976, respectively.

To test the effect of db-cAMP, non-everted intestinal segments were filled (mucosal compartment) with Krebs–Henseleit buffer (KHB 40 mM glucose, pH 7.40), and immersed in the serosal compartment consisting of 7 mL of KHB alone (control group) or 7 mL KHB containing 100 µM db-cAMP (db-cAMP group), 1 µM KT5720 (KT5720 group), or 100 µM db-cAMP + 1 µM KT5720 (db-cAMP + KT5720 group). The intestinal sacs were incubated for 30 min at 37 °C. Mucosal compartment from KT5720 and db-cAMP + KT5720 groups were also added 1 µM KT5720. In all cases, the external (serosal) compartment was continuously gassed with 95% O_2/5% CO_2. Preliminary experiments to demonstrate Mrp2 translocation were performed using 10 and 100 µM db-cAMP. As the 10 µM concentration failed to produce any effect (data not shown), we have chosen the 100 µM concentration for all further experiments in rat intestinal sacs. This concentration is in accordance with that used in previous studies demonstrating cAMP-induced Mrp2 sorting to the plasma membrane in rat hepatocytes (Schonhoff et al. 2010).

To test the effect of $E_2$17G, non-everted intestinal segments were filled (mucosal compartment) with KHB (40 mM glucose, pH 7.40) alone (control group), or with KHB containing 400 µM $E_2$17G ($E_2$17G group), 1 µM Gö6976 (Gö6976 group), or 400 µM $E_2$17G + 1 µM Gö6976 ($E_2$17G + Gö6976 group). The intestinal sacs were immersed in 7 mL of KHB (serosal compartment) and incubated for 30 min at 37 °C. Serosal compartment from Gö6976 and $E_2$17G + Gö6976 groups were also added 1 µM Gö6976. In all cases, the external (serosal) compartment was continuously gassed with 95% O_2/5% CO_2. Preliminary experiments to demonstrate Mrp2 translocation were performed using 100 and 200 µM of $E_2$17G in addition to the 400 µM concentration. The 100 µM concentration failed to produce any effect, whereas the 200 µM produced modest, not statistically significant effects (data not shown). In consequence, we have chosen the 400 µM concentration for all further experiments in rat intestinal sacs.

At the end of incubations, the intestinal sacs were carefully rinsed with ice-cold saline and opened lengthwise. Total homogenates were prepared using a 50 mM mannitol, 2 mM Tris, pH 7.10 buffer supplemented with protease inhibitors (25 mg/mL leupeptin, 40 mg/mL PMSF and 0.5 mg/mL pepstatin A) as described (Mottino et al. 2000). BBM fractions were obtained from total homogenates using a Ca^{2+}-precipitation method (Kessler et al. 1978) with some modifications (Mottino et al. 2000). Briefly, total homogenates were incubated with 10 mM $CaCl_2$ for 15 min and then subjected to differential centrifugation (15 min at 3000*g*, 4 °C and 40 min at 27,000*g*, 4 °C). To obtain the IM fraction, the last supernatant was centrifuged at 100,000*g* for 60 min at 4 °C as previously done by Kubitz et al. (2005) to prepare IM from liver tissue. The pellet (IM) was resuspended in appropriate volumes of 300 mM mannitol, 10 mM HEPES, 10 mM Tris, pH 7.50 buffer, supplemented with protease inhibitors. Preparations were subjected to protein concentration determination (Bradford 1976) and immediately used in Western blot analysis.

Assessment of Mrp2 activity

Evaluation of Mrp2 activity in everted intestinal sacs was performed as described by Mottino et al. (2001) with minor modifications. The model of everted sacs was chosen since it allows for taking samples from the mucosal compartment at different incubation times. To study the effect of db-cAMP on Mrp2 transport activity, the everted intestinal sacs were filled (serosal compartment) with KHB containing db-cAMP (100 µM) and immersed in the mucosal compartment consisting of 7 mL of KHB. After 30 min of incubation at 37 °C, CDNB, the precursor of the Mrp2 substrate DNP-SG, was added to the mucosal compartment at a 100 µM concentration and further incubated for 15 min. Aliquots from this same compartment were taken at 0, 5, 10 and 15 min. Then, intestinal sacs were dried and weighed. Identical experiments were repeated with addition of 20 µM MK571 (MRP inhibitor) together with CDNB (Lindenmaier et al. 2005). To study the effect of $E_2$17G on Mrp2 transport activity, everted intestinal sacs were filled (serosal compartment) with KHB and immersed in the mucosal compartment consisting of 7 mL of KHB containing 400 µM $E_2$17G. After 30 min of incubation at 37 °C, CDNB (100 µM) was added to the mucosal compartment and incubated for additional 15 min. Aliquots from this same compartment were taken at 0, 5, 10 and 15 min. Experiments were repeated with addition of the MRPs inhibitor MK571 (20 µM) into the mucosal compartment at the same time of CDNB incorporation.

All collected aliquots were deproteinized and subjected to quantification of DNP-SG and its derivative dinitrophenyl-cysteinyl glycine (DNP-CG) by HPLC as previously described (Hinchman et al. 1991). DNP-CG is produced by the action of γ-glutamyltransferase on DNP-SG at the BBM after this compound reaches the mucosal compartment (Hinchman et al. 1991). Therefore, the excretion rate of DNP-SG + DNP-CG properly estimates Mrp2 activity.

Experiments in Caco-2 cells

Cell culture

Caco-2 cell line was purchased from the American Tissue Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum (Natocor), 2 mM L-glutamine, a mixture of antibiotics (5 mg/mL penicillin, 5 mg/mL streptomycin and 6 ng/mL amphotericin B) and modified Eagle

medium nonessential amino acids (Life Technologies). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Culture medium was replaced every second day.

Assessment of MRP2 distribution between plasma (PM) and intracellular (IM) membrane fractions

For MRP2 localization studies, cells were seeded in 6-well plates (2.5×10^5 cells/well) and cultured for 15 days. After that, culture medium was replaced by fresh medium containing 10 µM db-cAMP or 400 µM $E_2$17G and incubated for 30 min at 37 °C. Control cells were exposed to vehicle (DMSO, 0.1% V/V). As for intestinal sac experiments, to assess the participation of PKA or cPKC in db-cAMP and $E_2$17G effects, incubations were additionally performed in the presence of 1 µM KT5720 or 1 µM Gö6976, respectively. Also, preliminary experiments were performed using 10 and 100 µM of db-cAMP and 100, 200 and 400 µM of $E_2$17G to establish the minimal concentration of these agents to produce a significant effect.

At the end of the incubations, PM and IM were obtained by differential centrifugation as described by Kubitz et al. (2005). Briefly, Caco-2 cells were washed twice, scraped and collected in 250 mM sucrose, 5 mM EGTA, 1 mM $MgCl_2$, 20 mM Tris, pH 7.40 buffer supplemented with protease inhibitors, and lysed by passing 20 times through a 25-G needle. The PM fraction was obtained by centrifugation at 1000*g* for 5 min at 4 °C. The resulting supernatant was centrifuged at 100,000*g* for 60 min at 4 °C to obtain the IM fraction.

Assessment of MRP2 activity

For MRP2 activity studies, cells were seeded in 96-well plates (9.0×10^3 cells/well) and cultured for 15 days. After that, culture medium was replaced by fresh medium containing 10 µM db-cAMP, 400 µM $E_2$17G, or vehicle (DMSO, 0.1% V/V). After 30 min of incubation at 37 °C, MRP2 activity was assessed as previously described (Arias et al. 2014). Briefly, after preloading with 100 µM DNP-SG for 30 min, cells were washed twice and incubated with Hank's balanced salt solution (HBSS) at 37 °C for 15 min. Then, medium and cell lysate were collected, deproteinized and subjected to DNP-SG quantification by HPLC as described (Hinchman et al. 1991). Incubations were repeated in the presence of 20 µM MK571, as inhibitor of MRPs. MRP2 activity was determined by quantifying the efflux of DNP-SG into the incubation medium referred to the total amount of DNP-SG available to be extruded. The total amount of DNP-SG available to be extruded was estimated as the sum of the amount of DNP-SG + DNP-CG accumulated in the medium and the amount of DNP-SG remaining in the cells at the end of the incubation.

Western blot studies

Once prepared, BBM, PM and IM fractions were immediately used in Western blot studies as previously described (Mottino et al. 2000). MRP2, (M_2III-6, Enzo Life Science), villin and CYP3A4 (H-60 and HL3; Santa Cruz Biotechnology) and β-actin (AC-74, Sigma-Aldrich) were used as primary antibodies. Immunoreactive bands were detected using a chemiluminescence kit (Pierce™ ECL Western Blotting Substrate, ThermoScientific) and quantified using ImageJ software.

Confocal microscopy studies

For immunodetection of MRP2, Caco-2 cells were cultured for 15 days in cover slips and treated with 10 µM db-cAMP or 400 µM $E_2$17G for 30 min. After the treatment, cells were fixed, permeabilized and blocked as previously described (Arana et al. 2015). MRP2 and Zonula Occludens-1 (ZO-1) were detected with same primary antibodies used in Western blot studies, followed by incubation with red and green fluorescence-labeled secondary antibodies, respectively (Arana et al. 2015). Nuclei were detected using 4,6-diamidino-2-phenylindole (DAPI). All images were collected with a Nikon C1 Plus confocal on a Nikon TE2000 inverted microscope equipped with a 60 × Plan Apo NA 1.4 objective. Optical Sections (23–31 z-series) were collected with a step size of 0.4 µm. Images are shown as top and 3D orthogonal views.

Statistical analysis

All results are expressed as mean ± standard deviation. Statistical comparisons were performed through one-way ANOVA followed by Tukey post hoc test, except for the study of rat intestinal Mrp2 activity vs. time (Fig. 2a) in which two-way repeated measures ANOVA followed by Tukey post hoc test was used. Statistical significance was set at $p < 0.05$.

Results

Modulation of Mrp2 localization and activity in rat jejunum

We initially characterized the distribution of Mrp2 between BBM and IM under normal conditions. Figure 1a shows that Mrp2 follows a similar pattern of distribution as villin, an integral protein of the BBM of mature enterocytes.

In contrast, it is present to a much lesser extent in the IM fraction, whose intracellular nature was confirmed by CYP3A4 enrichment. The data strongly suggest that Mrp2 is preferentially recovered with the apical membrane fraction, whereas only a small fraction of total Mrp2 is found in intracellular compartments. This agrees well with a previous immunohistochemical study showing a major signal on the surface of the villi from rat duodenum and jejunum (Mottino et al. 2000), and with more detailed studies performed by confocal microscopy demonstrating localization at the BBM of the enterocyte, in the vicinity of the tight junction protein ZO-1 (Arias et al. 2009). Whether IM content of Mrp2 corresponds to a subapical reservoir, transit from endoplasmic reticulum of newly synthesized protein, or both, was not determined. Taken together, the data demonstrate that the current fractionation methodology seems to be appropriate to detect eventual changes in response to the same agents previously known to induce insertion or retrieval in

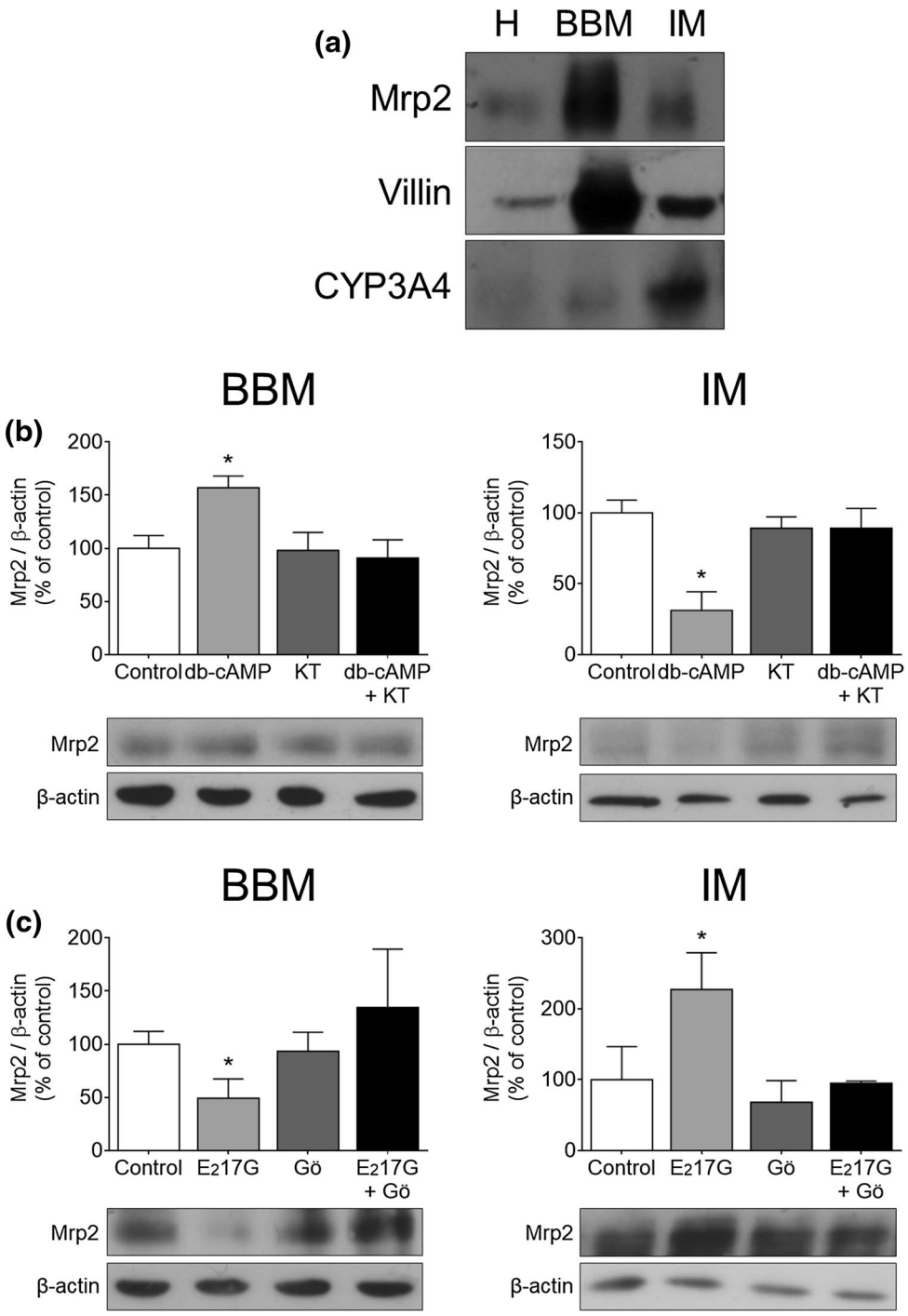

Fig. 1 Distribution of Mrp2 between BBM and IM in rat jejunum and its modulation by db-cAMP and $E_2$17G. **a** Representative blots for recovery of Mrp2, villin and CYP3A4 in plasma vs. intracellular membrane fractions are shown. Total homogenate (H), brush border (BBM) and intracellular membrane (IM) fractions from rat jejunal mucosa were used in Western blot studies. Uniformity of protein loading and transfer from gel to PVDF membrane were controlled with Ponceau S. Villin and CYP3A4 were used as BBM and IM markers, respectively. Same distribution of Mrp2, villin and CYP3A4 was confirmed in three independent experiments. **b**, **c** Representative blots for detection of Mrp2 in brush border membrane (BBM) and intracellular membrane (IM) fractions after db-cAMP and $E_2$17G treatments are shown together with the respective densitometric analysis. Involvement of PKA (**b**) and cPKC (**c**) was studied using the respective inhibitors Gö6976 (Gö) and KT5720 (KT). Uniformity of protein loading and transfer from gel to PVDF membranes were controlled with Ponceau S and detection of β-actin. Data are presented as % of control group and expressed as mean ± standard deviation ($n = 4$). *Different from control group, $p < 0.05$

different liver experimental models. We first evaluated the acute effect (30 min) of the permeable analog of cAMP, db-cAMP, on Mrp2 distribution between BBM and IM. Figure 1b shows an increase in the apical content of Mrp2 with respect to the control group, and a concomitant decrease in IM content, consistent with rapid translocation from an intracellular compartment to the BBM. Since db-cAMP is a direct activator of PKA, we evaluated whether this kinase is involved in stimulation of Mrp2 insertion using the selective inhibitor KT5720. As shown in the same figure, the effect of db-cAMP was completely abolished by the PKA inhibitor, confirming involvement of a PKA-dependent mechanism. Figure 1b also shows that KT5720 itself had no effect on Mrp2 distribution.

We also evaluated the effect of $E_2$17G, a cholestatic endogenous metabolite of estradiol previously shown to induce Mrp2 internalization in rat liver (Mottino et al. 2002). Figure 1c shows that $E_2$17G induced a decrease in Mrp2 content in BBM fraction with respect to the control group and a concomitant increase in IM fraction, consistent with an endocytic internalization of the transporter. Endocytosis of Mrp2 was blocked by the selective cPKC inhibitor Gö6976, confirming the participation of this kinase. The same figure shows that Gö6976 alone did not affect Mrp2 localization.

To establish whether the above-described changes in Mrp2 localization are associated with changes in transport activity, we evaluated the rate of secretion of the model Mrp2 substrate, DNP-SG. Figure 2a shows that db-cAMP treatment led to a significant increase in DNP-SG accumulation in the mucosal compartment, which reached significance at 10 and 15 min. The increase was of 33% over the control group when expressed as amount of DNP-SG accumulated after 15 min of incubation (Fig. 2b). In contrast and as expected from data on Western blot studies, short-term treatment with $E_2$17G resulted in a significant decrease in DNP-SG accumulation in the mucosal compartment at all time points evaluated (Fig. 2a). This is clearly visualized after analyzing the amount of DNP-SG accumulated at 15 min (Fig. 2b), which was decreased by 38% with respect to controls. Transport activity was also assessed in these same groups in the presence of the MRPs inhibitor MK571. The significant decrease detected in all groups in comparison with transport activity found in absence of the inhibitor, confirms that a MRP protein is at least partially involved in DNP-SG secretion in normal and treatment conditions (Fig. 2b).

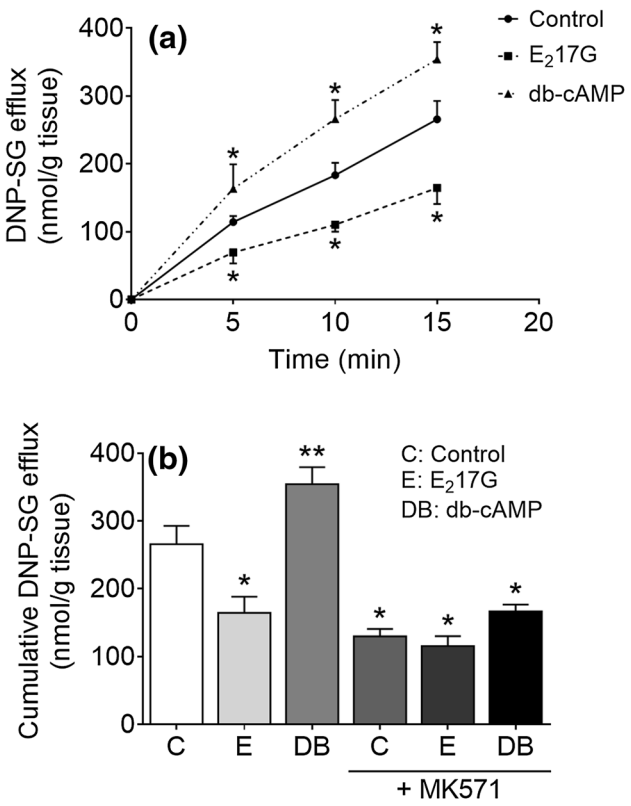

Fig. 2 Modulation of Mrp2 activity by db-cAMP and $E_2$17G in isolated rat intestinal sacs. Time course of DNP-SG efflux into the mucosal compartment (**a**) and DNP-SG accumulation into the same compartment after 15 min of incubation (**b**). The effect of the MRP inhibitor MK571 is shown in **b**. Data are expressed as mean ± standard deviation ($n = 4$). *Different from control group, $p < 0.05$. **Different from all other groups, $p < 0.05$

Modulation of MRP2 localization and activity in Caco-2 cells

Only few studies explored the short-term regulation of Mrp2 activity in small intestine from laboratory animals. Even less information is available on its regulation in human intestine or in human intestinal cell lines. We here evaluated the effect of db-cAMP and $E_2$17G on MRP2 localization in Caco-2 cells, a model of human intestinal epithelium. We first checked the efficiency of a membrane fractionation method previously found to be appropriate to separate PM from IM in liver tissue (Kubitz et al. 2005). Figure 3a shows that villin is clearly enriched in PM whereas CYP3A4 is recovered mainly in IM. MRP2 is recovered in both fractions, though a slightly higher content is found in PM vs. IM. According to these data, it is possible to speculate that a higher proportion of transporter protein is found intracellularly in Caco-2 cells than in rat jejunum. Localization of MRP2 was further studied in situ by confocal microscopy. Figure 4 shows images of Caco-2 cells with fluorescent detection of MRP2 in red, ZO-1 in green and nuclei in blue. The upper, main image shows the structure of Caco-2 cell culture (top view), which is consistent with an established monolayer epithelium. Cells are delimited by tight junction structures identified by ZO-1 signal. Images created by stacking along

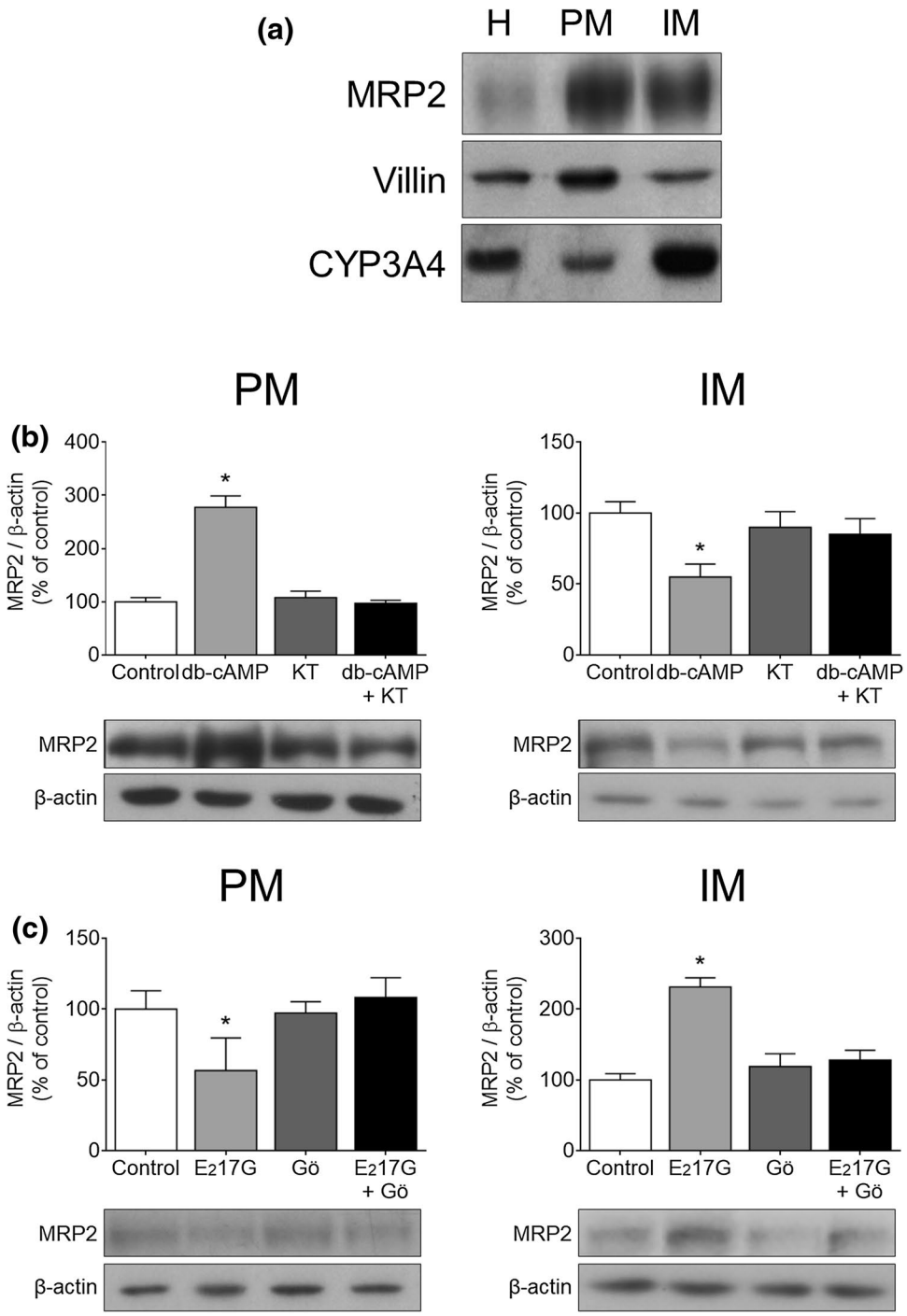

Fig. 3 Distribution of MRP2 between PM and IM in Caco-2 cells and its modulation by db-cAMP and $E_2$17G. **a** Representative blots for recovery of MRP2, villin and CYP3A4 in plasma vs. intracellular membrane fractions are shown. Total cell lysate (H), plasma (PM) and intracellular membrane (IM) fractions from Caco-2 cells were used in Western blot studies. Uniformity of protein loading and transfer from gel to PVDF membrane were controlled with Ponceau S. Villin and CYP3A4 were used as PM and IM markers, respectively. Same distribution of MRP2, villin and CYP3A4 was confirmed in three independent experiments. **b**, **c** Representative blots for detection of MRP2 in plasma membrane (PM) and intracellular membrane (IM) fractions are shown together with the respective densitometric analysis. Involvement of PKA (**b**) and cPKC (**c**) was studied using the respective inhibitors Gö6976 (Gö) and KT5720 (KT). Uniformity of protein loading and transfer from gel to PVDF membranes were controlled with Ponceau S and detection of β-actin. Data are presented as % of control group and expressed as mean ± standard deviation ($n = 4$). *Different from control group, $p < 0.05$

the *Z*-axis are shown at the right and bottom of the top view image. A small region of the *Z*-axis image is shown with higher magnification and demonstrates that MRP2 was detected on the cell surface and also intracellularly, in agreement with the Western blot studies of PM and IM; a B&W image of the *Z*-axis stacking is shown exclusively for the red channel (MRP2) at the bottom of Fig. 4 and confirms such distribution. When Caco-2 cells were incubated with db-cAMP for 30 min, a significant increase in MRP2 recovery in PM fraction and a concomitant decrease in IM were observed with respect to control cells (Fig. 3b). The pattern of distribution of MRP2 and ZO-1 was undistinguishable from that of control cells in the confocal top view (Fig. 4). However, the stacking along the *Z*-axis evidences that MRP2 signal was mainly concentrated on the apical surface of the cell layer, with no apparent changes in the pattern of ZO-1 distribution. Comparison between B&W images from control and db-cAMP-treated cells

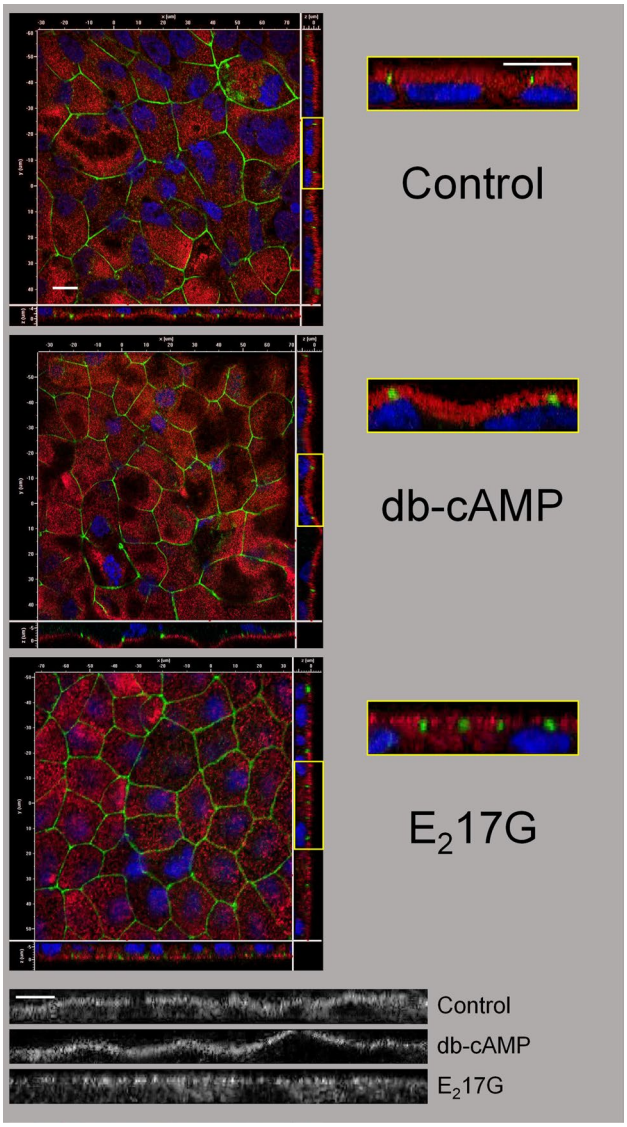

Fig. 4 Confocal microscopy detection of MRP2 in Caco-2 cells. Main images represent top view of the monolayer culture, together with images created by stacking along the Z-axis, which are shown at the right and bottom of the top view images. A small region of the Z-axis image from each group is shown with higher magnification on the right. Tight junction protein ZO-1 was labeled with green fluorescence and MRP2 with red fluorescence. Nuclei were detected with DAPI (blue fluorescence). B&W images at the bottom reproduce the stacking along the Z-axis (those on the right of top view images) exclusively for the red channel (MRP2). Scale bars represent 10 µm

illustrates well that detection of MRP2 was restricted to the BBM. Although no quantitative analysis was performed on confocal images, taken together the data suggest increased sorting of MRP2 to the PM. Western blot studies performed in PM and IM from cells treated with the PKA inhibitor KT5720 demonstrated that the effect previously shown for db-cAMP was completely abolished. No change was found in MRP2 distribution between PM and IM after incubation with the inhibitor alone.

In contrast to db-cAMP effects, $E_2$17G decreased MRP2 content in PM fraction and increased its expression in IM fraction when compared to control cells (Fig. 3c). Confocal images show that distribution of MRP2 along the *Z*-axis was also affected in response to $E_2$17G. The *Z*-axis stacking in Fig. 4 evidences increased the presence of MRP2 in the cytosol when compared to control cells, consistent with internalization of MRP2. It is worth noting that the pattern of distribution of MRP2 differed from those of control and db-cAMP groups, as it shows a punctate staining. The nature of such particular distribution is unknown, but could reflect endocytic vesiculation leading to loss of MRP2 from specific membrane regions. Image in B&W supports such possibility as MRP2 signal shows intermittencies at the BBM level, contrasting with the more continuous staining in control and db-cAMP groups. While incorporation of the cPKC inhibitor Gö6976 alone to the incubations did not affect the localization of MRP2 between PM and IM as detected by Western blotting, it was able to prevent the redistribution induced by $E_2$17G when incorporated together with the cholestatic agent (Fig. 3c).

The functional impact of changes in MRP2 localization induced by db-cAMP and $E_2$17G was also evaluated. Figure 5 shows that db-cAMP-treated cells exhibited increased MRP2 activity (17%) when compared to control cells. In contrast, $E_2$17G induced a decrease in this measure by 24%. Incorporation of the MRP inhibitor MK571 to the incubations prevented such modifications to the extent that there were no differences between groups.

Discussion

MRP2 is expressed in small intestine according to a gradient with the highest levels in proximal jejunum and a decrease towards the distal ileum (Mottino et al. 2000). It plays a significant role in protecting the enterocytes from chemical injury and, more importantly, in preventing absorption of toxicants such as food contaminants. Well known examples of MRP2 substrates of toxicological relevance are the mycotoxin ochratoxin A (Schrickx et al. 2006) and the most abundant heterocyclic amine in cooked meat PhIP (Dietrich et al. 2001). Modulation of expression of MRP2 either under pathophysiological conditions or in response to xenobiotics, including drugs of therapeutic use, results in long-term regulation of MRP2-dependent barrier function (Gerk and Vore 2002; Arana et al. 2016; Tocchetti et al. 2016). An acute regulation of MRP2 activity is also possible, resulting in rapid modification of its barrier function. Whereas short-term regulation of ABC transporters based on changes in localization between plasma membrane and intracellular

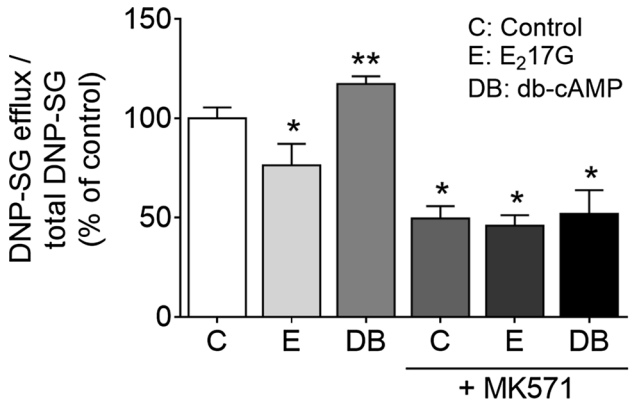

Fig. 5 Modulation of MRP2 activity by db-cAMP and $E_2$17G in Caco-2 cells. The activity of MRP2 was determined through calculation of the relationship between the amount of DNP-SG (DNP-SG + DNP-CG) accumulated in the medium after 30 min of incubation and the total amount of DNP-SG available to be extruded, in the presence or absence of MK571. Data are presented as % of control group and expressed as mean ± standard deviation ($n = 4$). *Different from control group, $p < 0.05$. **Different from all other groups, $p < 0.05$

compartments has been well characterized in liver, studies in intestine are limited. We here provide evidence supporting the existence of such regulation in both directions, i.e., increased sorting to, and retrieval from the BBM. It is worth noting that this bidirectional regulation occurred irrespective of the initial distribution of MRP2 between PM and IM, which was clearly different between models. In addition, the effects described for the two agents currently studied, db-cAMP and $E_2$17G, may have not only toxicological but also physiological and pathophysiological implications.

We first evaluated the effect of db-cAMP and found that it stimulated the insertion of MRP2 into the plasma membrane in both intestinal sacs and Caco-2 cells. The effect was accompanied by increased secretory activity of the model substrate DNP-SG, consistent with the notion that a proper localization at the apical membrane of secretory epithelia is essential. cAMP constitutes a ubiquitous intracellular messenger involved in the action of different hormones, growth factors and neurotransmitters, as well as a wide spectrum of xenobiotics including drugs of therapeutic use. cAMP is produced by adenylyl cyclase, which in association with G-protein-coupled receptors represent the target of several hormones including ligands of the glucagon superfamily of receptors; GLP-2 is one of these ligands. GLP-2 is mainly produced at the distal small intestine by specialized, L-type endocrine cells (Ørskov et al. 1986). It is implicated in the regulation of intestinal growth and absorptive function under physiological conditions such as development (Drucker 2002) or lactation (Jacobs et al. 1981). Since the presence of GLP-2 receptor (GLP-2R) has not been confirmed in enterocytes, it is not clear how the hormone exerts its regulatory function in these cells. It was proposed that other cell types expressing the receptor communicate with enterocytes through unidentified paracrine factors or neurotransmitters (Rowland and Brubaker 2011). In a previous study we demonstrated that repeated administration of GLP-2 to rats induced an increase in Mrp2 expression in mature enterocytes in a cAMP-dependent fashion (Villanueva et al. 2010). In a more recent study we have demonstrated that increased levels of cAMP in Caco-2 cells resulted in up-regulation of MRP2 expression through a PKA-mediated mechanism (Arana et al. 2015), thus linking findings on GLP-2-induced up-regulation of MRP2 with the participation of cAMP and PKA. A question arises on whether transient secretion of GLP-2 occurring shortly after nutrients incorporation into the intestinal lumen may trigger short-term regulation of MRP2 activity, e.g., by stimulating insertion into the BBM. Our current findings support such possibility. The whole sequence of events would be (1) nutrients triggers secretion of GLP-2 (Dubé and Brubaker 2004), (2) a direct or indirect action of GLP-2 increases cAMP levels in the enterocyte (Villanueva et al. 2010), and (3) high cAMP intracellular levels stimulate sorting of MRP2 to the BBM. If true, this would constitute a novel, on-demand regulation of the transcellular barrier function associated with MRP2 activity to protect against cell toxicity and absorption of food contaminants during absorptive periods. Although this hypothesis needs further, more direct, demonstration, other authors have already demonstrated that MRP2 can be regulated in a short-term fashion in response to specific requirements. One interesting example is the rapid increase of MRP2 expression at the apical surface of HCT8 human intestinal cells in response to *Salmonella typhimurium* infection, which resulted in increased efflux of the potent neutrophil chemoattractant, MRP2 substrate, hepoxilin A3 (Agbor et al. 2011). The obvious consequence is increased recruitment of leukocytes to the intestinal luminal surface to deal with bacterial infection.

As shown in Figs. 2a and 4a, the PKA inhibitor KT5720 completely abolished redistribution of MRP2 induced by db-cAMP irrespective of the experimental model. The intracellular mechanisms subjacent to PKA activation were not studied. Several studies demonstrated microtubule-dependent insertion of MRP2 into the apical membrane in different models (Roelofsen et al. 1998; Roma et al. 2000; Mottino et al. 2005). Reestablishment of Mrp2 apical localization after oxidative stress challenge was also found to be PKA dependent and mediated by microtubules (Sekine et al. 2008). In this latter study, the authors proposed that motor molecules associated with microtubules such as kinesin2, myosin5 or dynein could be implicated. Additionally, Schonhoff et al. (2010) demonstrated participation of p38α MAPK in cAMP-stimulated sorting of MRP2 to the plasma membrane of HuH-7 human liver cells. Similar mechanisms

could be implicated in increased sorting of MRP2 to the BBM in response to cAMP.

The effect of the endogenous derivative of estradiol, $E_2$17G, was also evaluated in the present study. The data indicate that MRP2 was retrieved from the plasma membrane to intracellular compartments, with concomitant loss of transport activity. A similar effect was previously demonstrated in rat liver (Mottino et al. 2002) and isolated hepatocyte couplets (Crocenzi et al. 2008). The pathophysiological relevance of these findings remains to be demonstrated. However, $E_2$17G is expected to increase significantly in bile during pregnancy, estrogen replacement therapy or contraception treatments (Vore et al. 1997). Meyers et al. (1980) administered increasing doses of [^{3}H] $E_2$17G i.v. to rats and found that about 80% of the administered radioactivity was excreted in bile along a 3-h period, with intact $E_2$17G present in substantial amounts. More importantly, [^{3}H] estrogen derivatives, including $E_2$17G, were significantly concentrated and secreted in bile before reaching cholestatic levels, which suggests that the surface of the intestine could be certainly exposed to high amounts of $E_2$17G of biliary origin. Unfortunately, no studies evaluated the levels of $E_2$17G in bile from pregnant women or rats, or during estrogen replacement therapy or contraceptive administration.

In a previous study, we administered $E_2$17G i.v. following the acute, cholestatic protocol (Mottino et al. 2002), and evaluated the effect on intestinal Mrp2 transport activity and cellular localization in the enterocyte by confocal microscopy. Under that protocol, and due to i.v. administration, $E_2$17G was expected to reach the intestinal epithelium only from the serosal side. The data showed no changes in cellular localization of Mrp2, whereas its transport activity was significantly impaired, which was attributed to competitive inhibition of DNP-SG transport by $E_2$17G (Arias et al. 2009). In the current protocol, $E_2$17G was incorporated to the mucosal/apical side and resulted in significant retrieval of MRP2 irrespective of the experimental model. Lack of effect on Mrp2 localization in the previous study may have resulted from insufficient dose of $E_2$17G, from inaccessibility to the enterocytes, or both. Unfortunately, in our experimental conditions, it is not possible to distinguish if impairment in DNP-SG secretion depends exclusively from loss of apical MRP2 or if competitive inhibition by $E_2$17G is also present. In any case, the current study suggests that high concentrations of $E_2$17G acting from the intestinal lumen may have a significant impact on MRP2 activity and consequently on its barrier function.

Results in Figs. 2b and 4b demonstrate a role for cPKC in endocytic internalization of MRP2 induced by $E_2$17G in intestinal sacs and Caco-2 cells. Involvement of cPKC in $E_2$17G effects was somehow expected since it was previously found to mediate vesicular internalization of Mrp2 in rat hepatocytes (Crocenzi et al. 2008). Phosphorylation of members of the ERM complex like radixin could be involved, at least partially, in retrieval of hepatic MRP2 induced by protein kinases (Anwer 2014). In this regard, Sekine et al. (2011) found that cPKC activation under oxidative stress conditions resulted in decreased interaction of liver Mrp2 with radixin, ultimately leading to retrieval from the canalicular membrane. In intestine, the phosphorylation status of ezrin, modulated by cPKC in thymeleatoxin-treated rats, has been causally linked to retrieval of Mrp2 as a consequence of decreased protein–protein interaction between ezrin and Mrp2 (Nakano et al. 2009). Further experiments are needed to identify the cPKC member/s mediating MRP2 retrieval as currently seen, and whether ERM proteins, or MRP2 itself, are the phosphorylation targets.

Caco-2 cell cultures are among the several preclinical models of frequent use to predict human in vivo permeability of drugs, which also include rat jejunum perfused in situ and fragments of human intestinal segments mounted on using chambers (Lennernäs 1997; Dahlgren et al. 2015). Passively transported compounds can be predicted with acceptable accuracy, whereas prediction of permeability must be cautiously done for drugs subjected to carrier-mediated transport. Our findings in Caco-2 cells demonstrated that MRP2 is expressed at the apical membrane and also intracellularly, and that its translocation may occur in both directions. We additionally demonstrated that agents of well-established actions on the liver similarly affect MRP2 localization in Caco-2 cells. Of particular interest is the finding on changes in the pattern of MRP2 distribution in response to $E_2$17G observed in confocal studies. This guarantees further exploration to identify the nature of such redistribution and in consequence, the mechanism involved. To our knowledge this is the first study characterizing constitutive distribution of MRP2 between PM and IM in this cell model, either by membrane fractionation or in situ examination, as well as its short-term bidirectional regulation.

In conclusion, we demonstrate redistribution of intestinal MRP2 between PM and IM in rat jejunum and in Caco-2 cells in response to db-cAMP and $E_2$17G, with PKA and cPKC, respectively, involved as mediators. MRP2 redistribution was associated with changes in its transport activity. Taken together, the data suggest that localization of MRP2 in intestine may be subjected to a dynamic equilibrium between plasma membrane and intracellular domains, thus allowing for rapid regulation of MRP2 function.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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