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Selective upregulation of the expression of plasma membrane calcium ATPase isoforms upon differentiation and 1,25(OH)$_2$D$_3$-vitamin treatment of colon cancer cells

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ABSTRACT

We have previously presented co-expression of the plasma membrane calcium ATPase isoforms 4b (PMCA4b) and 1b (PMCA1b) in colon carcinoma cells, and selective upregulation of PMCA4b during differentiation initiated by short chain fatty acids or post-confluent growth. Here we show that the induction of PMCA4b expression is a characteristic feature of the post-confluency-induced differentiation of both enterocyte-type and goblet cell-type colon cancer cells. Vitamin D$_3$ (1,25(OH)$_2$D$_3$) is a well-known regulator of intestinal Ca^{2+} absorption and of basic cell functions such as growth and differentiation in various cell types. As PMCA proteins are involved both in intestinal Ca^{2+} absorption and adenocarcinoma cell differentiation, we investigated the effect of 1,25(OH)$_2$D$_3$ on PMCA expression in enterocyte-like colon carcinoma cells, and monitored its effect on the expression of various differentiation markers. 1,25(OH)$_2$D$_3$ stimulated PMCA1b, but not PMCA4b expression without modulating the expression of the majority of the differentiation markers examined. Caco-2 cells differentiated in post-confluent cultures present normal enterocyte-like intestinal epithelial phenotype. To better understand the role of PMCA proteins in vectorial Ca^{2+} transport by enterocytes, we also studied their subcellular localization in mature polarized Caco-2 cells. Both PMCA isoforms were located to the basolateral membrane, and the PMCA-specific immunofluorescent signal was significantly higher in vitamin D$_3$-treated cells, underlining the 1,25(OH)$_2$D$_3$-induced upregulation of PMCA (presumably 1b isoform) expression in differentiated Caco-2 cells. We suggest that while PMCA1b has a housekeeping function in colon cancer cells, PMCA4b participates in the reorganization of the Ca^{2+} signalling machinery during cell differentiation. The subcellular localization of PMCA1b and its selective 1,25(OH)$_2$D$_3$-dependent upregulation indicate that this isoform may have a specific role in 1,25(OH)$_2$D$_3$-stimulated intestinal Ca^{2+} absorption.

1. Introduction

Signal transduction pathways regulated partly by cellular Ca^{2+} signalling control several cell functions including cell growth and differentiation [1]. Plasma membrane calcium ATPases (PMCAs) are essential for the adjustment of the resting cytoplasmic Ca^{2+} concentration and for the regulation of global and dynamic Ca^{2+} signals [2]. Accumulating data indicate that the expression and/or activity of Ca^{2+} transport proteins, including PMCA pumps, may

Abbreviations: 1,25(OH)$_2$D$_3$, 1,25-dihydroxycholecalciferol, calcitriol; DPP-IV, dipeptidyl peptidase-IV; DMEM, Dulbecco's modified Eagle's Medium; DPBS, Dulbecco's modified PBS; PMCA, plasma membrane calcium ATPase; SERCA3, sarco/endoplasmic reticulum calcium ATPase 3; SCFA, short chain fatty acid.

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regulate cell maturation [3,4]. Furthermore, modulation of PMCA expression has been observed during the differentiation of different cell types [5–11].

Human PMCA proteins are encoded by 4 genes (*PMCA1-4* or *ATP2B1-4*), and alternative splicing of the primary transcripts generates a multitude of PMCA isoforms [12]. We have previously shown that PMCA1b and PMCA4b proteins are simultaneously expressed in colon and breast carcinoma cells, and that the induction of cell differentiation resulted in characteristic changes of the PMCA expression pattern [8,11]. Induction of PMCA4b expression during the differentiation of MCF-7 breast carcinoma cells leading to enhanced calcium efflux has also been described [11].

Under normal physiological conditions intestinal/colonic epithelial cells are continuously and rapidly renewed. Stem cells located at the base of the crypts of Lieberkühn stop to proliferate and begin to differentiate as they migrate from the crypt base towards the surface. Altered regulation of these proliferative and differentiation processes may lead to the formation of benign or malignant tumours. Short chain fatty acids (SCFAs), such as butyrate, physiologically present in the colonic lumen due to bacterial fermentation of dietary fibres, have anti-tumour activity as they inhibit growth and induce differentiation of *in situ* premalignant lesions [13]. Although expressed in normal epithelium, PMCA4 protein levels are decreased in colon tumours [14]. Importantly, our previous results demonstrated that *in vitro* differentiation of various colon and breast tumour cell lines, initiated by physiologically relevant concentrations of SCFAs resulted in a marked induction of PMCA4b expression, whereas PMCA1b expression was not affected, or was only moderately increased [8,11]. Furthermore, the expression of PMCA4b was also induced during the post-confluent differentiation of Caco-2 colon cancer cells [8]. In the present work we further analyse the expression profile of PMCA isoforms during the post-confluent differentiation of various colon adenocarcinoma cell lines of absorptive epithelial or mucus-secreting goblet cell phenotype.

$1,25(OH)_2D_3$-vitamin has a dual effect on the intestinal epithelium. By modulating both transcellular and paracellular Ca^{2+} transport, this vitamin is a key regulator of Ca^{2+} absorption that occurs mainly in the small intestine. In enterocytes $1,25(OH)_2D_3$ controls the expression of various Ca^{2+} transport and Ca^{2+} binding proteins essential for transcellular Ca^{2+} absorption [15]. Besides this widely accepted physiological role of $1,25(OH)_2D_3$, a series of epidemiological and nutritional data indicate that $1,25(OH)_2D_3$ has also chemopreventive effects on the malignancies of the intestinal tract [16,17]. Furthermore, data suggest that survival rates are linked to the $1,25(OH)_2D_3$ status of patients with colon cancer [16]. Although the anti-proliferative, differentiation- or apoptosis-inducing activities of $1,25(OH)_2D_3$ on various tumour cell types have been well documented [17], its effects on intestinal adenocarcinoma cells are poorly understood. Dietary Ca^{2+} is known to have tumour-preventive effects in the intestine. Thus, $1,25(OH)_2D_3$-modulated cellular Ca^{2+} homeostatic effects, as well as $1,25(OH)_2D_3$-regulated cell growth and differentiation may be connected/interrelated in intestinal epithelial and adenocarcinoma cells.

Since remodelling of cellular Ca^{2+} homeostasis seems to be an important event in the differentiation program of intestinal adenocarcinoma and epithelial cells, we compared differentiation-related changes in the PMCA expression pattern of *in vitro* models of two main cell types of intestinal epithelium, i.e., absorptive enterocytes and goblet cells. In enterocytic model cells we also investigated the effects of $1,25(OH)_2D_3$ on the localization and/or expression of various PMCA isoforms, as these proteins are potential actors in intestinal Ca^{2+} absorption, and may also participate in the control of cell growth and differentiation, all seeming to be under the regulation of $1,25(OH)_2D_3$.

2. Materials and methods

2.1. Cell cultures

Caco-2 and DLD-1 colon adenocarcinoma cell lines were obtained from the ATCC (Manassas, VA, USA), and cultured as described in Ref. [8]. The 5M12 and the 5M21 clones derived from the HT-29 colon adenocarcinoma cell line were generous gifts from Dr. T. Lesuffleur (INSERM, UMR S938, Paris, France) [18,19]. HT29-5M12 and HT29-5M21 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; High Glucose, GibcoBRL Life Technologies Ltd, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine. Because the 5M12 (enterocytic) and 5M21 (mucus-secreting, goblet cell-type) HT29-derived clones had been obtained earlier using selection in the presence of methotrexate [18,19], culture medium of these cells was also supplemented with 10 μM methotrexate in our experiments. All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Induction of cell differentiation in post-confluent cultures of Caco-2, DLD-1, HT29-5M12 and HT29-5M21 cells was performed as described for Caco-2 cells in Ref. [8].

2.2. Cell treatments

Pre-confluent, early (1 or 2 days) and late (20 days) post-confluent Caco-2 or DLD-1 cells were treated with 10 or 100 nM $1,25(OH)_2D_3$ every other day for 6 days. $1,25(OH)_2D_3$ (Sigma-Aldrich) dissolved in DMSO was added to the cell cultures from concentrated stock solutions. The final concentration of DMSO did not exceed 0.1%. DMSO was also employed in control experiments and did not interfere with the assays.

2.3. Western blot analysis

Western blot analysis using the PMCA4b-specific JA3 and the pan-PMCA-specific 5F10 monoclonal antibodies, or using antibodies against various differentiation markers (CEA, DPP-IV, SERCA3) was performed as described in Ref. [8]. The same protocol was used for the detection of PMCA1b expression with the PMCA1-specific NR1 antibody (dilution of 1:800) [20].

2.4. Quantitative evaluation of the changes in PMCA expression

The protocol described in Ref. [8] was used to estimate the fold increases in PMCA1b expression during $1,25(OH)_2D_3$ treatments of early post-confluent Caco-2 and DLD-1 cells. Changes in PMCA1b expression were determined using the mAb 5F10 antibody. As the PMCA1b and PMCA4b isoforms were clearly separated during electrophoresis, their expression levels could be independently studied.

2.5. Immunocytochemistry

Day 26 post-confluent Caco-2 cells treated with 100 nM $1,25(OH)_2D_3$ or vehicle (DMSO) were washed three times with Dulbecco's modified PBS (DPBS) and fixed with 4% paraformaldehyde for 5 min at 37 °C. After five washes with DPBS, cells were permeabilized with ice cold methanol for 5 min and washed five times again with DPBS. Subsequently, cells were incubated in blocking buffer (DPBS containing 2 mg/ml bovine serum albumin, 1% fish gelatin, 0.1% Triton-X 100, 5% goat serum) overnight at 4 °C, then subjected to mAb 5F10 (dilution 1:300) in the presence or absence of a chicken polyclonal anti-Na,K-ATPase antibody (Abcam, dilution 1:500) for 1 h at room temperature (RT). Parallel samples

were labelled with mouse IgG_{2a} (isotype control for mAb 5F10, Sigma-Aldrich) with or without chicken IgG (Sigma-Aldrich). After three washes with DPBS, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) secondary antibody or with the mixture of the Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) and Alexa Fluor 594-conjugated goat anti-chicken IgG (H + L) secondary antibodies (Life Technologies) for 1 h at RT. To visualize nuclei DAPI (Life Technologies) was used. Image acquisition was performed with an Olympus IX-81 laser scanning confocal microscope, using the Fluoview FV500 software v4.1.

3. Results

3.1. Post-confluency-induced differentiation of enterocyte- and goblet cell-type colon cancer cells results in marked changes of the PMCA expression profile

Our previous results demonstrated that *in vitro* differentiation induced by histone deacetylase inhibitors (*e.g.,* butyrate) significantly upregulated PMCA4b expression in various colon cancer cell lines. The expression of the housekeeping PMCA1 isoform, however, did not change or was only moderately increased in the differentiating adenocarcinoma cell lines examined [8]. Here, we monitored the expression of these PMCA isoforms during the post-confluency-induced differentiation of DLD-1, Caco-2, HT29-5M12 and HT29-5M21 colon cancer cells. Caco-2 and HT29-5M12 cells undergo enterocytic [18,19,21], whereas HT29-5M21 cells undergo goblet cell-type [19] differentiation. The post-confluent maturation of DLD-1 cells was found to be less complete as compared to that of the Caco-2 cell line. In our experiments the expression levels of two differentiation markers, the sarco/endoplasmic reticulum calcium ATPase 3 (SERCA3) and carcinoembryonic antigen (CEA) were elevated in both late post-confluent DLD-1 (data not shown) and Caco-2 cell cultures [8,22]. On the other hand, the expression of the differentiation marker dipeptidyl peptidase-IV (DPP-IV) was only enhanced in differentiated Caco-2 [8,22], but not in DLD-1 cells (data not shown). As shown in Fig. 1 (middle panels), the expression of PMCA4b highly increased during the differentiation of both enterocyte-type and goblet cell-type colon cancer cells. The expression of the PMCA1b isoform, on the other hand, (Fig. 1, left panels) increased only moderately during the maturation of Caco-2 cells, and did not change in differentiating DLD-1, HT29-5M12 and HT29-5M21 cells. Our results demonstrate that the modulation of the PMCA expression pattern leading to enhanced PMCA4b levels is a characteristic phenomenon during spontaneous differentiation of both enterocyte-type and goblet cell-type colon cancer cells.

3.2. Effect of 1,25(OH)$_2$D$_3$ on the expression of PMCA isoforms and of differentiation markers in colon cancer cells

The effect of 1,25(OH)$_2$D$_3$ on the expression of PMCA1b and PMCA4b was also monitored in pre-confluent (undifferentiated) and early post-confluent (differentiating) Caco-2 and DLD-1 colon cancer cells. 6 day long 1,25(OH)$_2$D$_3$ treatments (10–100 nM) had no effect on the expression of PMCA4b either in pre-confluent or in 1–2 day post-confluent Caco-2 and DLD-1 cells. In contrast, 10–100 nM 1,25(OH)$_2$D$_3$ treatments increased the expression of PMCA1b both in pre-confluent and in early post-confluent colon cancer cells (Fig. 2A–D). The regulatory effect of 1,25(OH)$_2$D$_3$ was more pronounced in differentiating *versus* undifferentiated Caco-2 and DLD-1 cells. The expression of PMCA1b was increased by 2- and 4-fold in early post-confluent Caco-2 and DLD-1 cells, respectively.

Next we tested the effects of post-confluent culturing *versus* 1,25(OH)$_2$D$_3$ treatments on the expression of various differentiation markers in Caco-2 cells (Fig. 2E,F). While post-confluent culturing for 26 days highly increased the expression of the intestinal brush-border hydrolase DPP-IV, the SERCA3, and the CEA differentiation markers in Caco-2 cells (Fig. 2E), 6 day long 10–100 nM 1,25(OH)$_2$D$_3$ treatments of pre-confluent cells had no such an effect. In day 2 post-confluent differentiating Caco-2 cells, 6 day long 10–100 nM 1,25(OH)$_2$D$_3$ treatments did not increase DPP-IV and SERCA3 expressions, but they elevated CEA expression (Fig. 2F). From these data we conclude that 1,25(OH)$_2$D$_3$ does not regulate the global differentiation process in Caco-2 cells.

3.3. 1,25(OH)$_2$D$_3$-dependent expression and localization of PMCA proteins in differentiated Caco-2 cells

Using confocal microscopy we investigated the subcellular localization of PMCA proteins in polarized, day 26 post-confluent Caco-2 cells cultured in the absence or presence of 100 nM 1,25(OH)$_2$D$_3$ from day 20 post-confluency. In both 1,25(OH)$_2$D$_3$-treated (Fig. 3B) and untreated (data not shown) differentiated Caco-2 cells PMCA proteins co-localized with the basolateral plasma membrane marker Na,K-ATPase. No apically localized PMCA pumps were detected. The 1,25(OH)$_2$D$_3$ treatment greatly enhanced the PMCA-specific immunofluorescent signal intensity as compared to that of the vehicle-treated cells (Fig. 3A). These data further confirm the regulatory effect of 1,25(OH)$_2$D$_3$ on PMCA (presumably PMCA1b) protein expression.

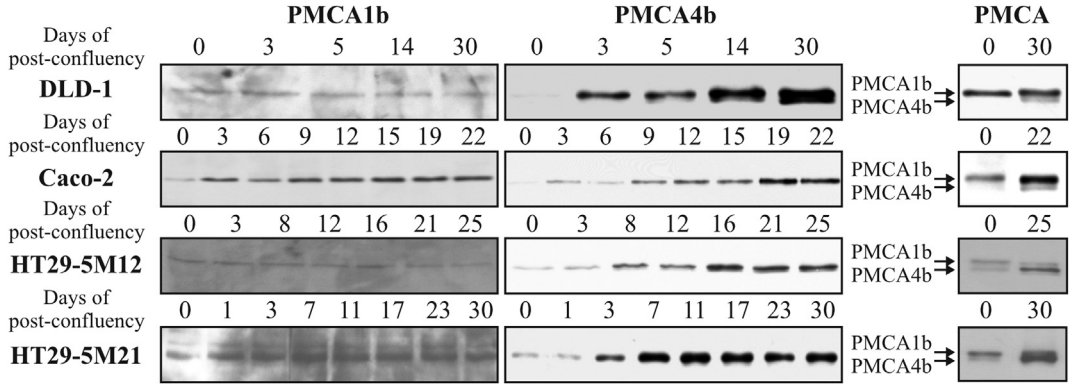

Fig. 1. Expression of PMCA isoforms during post-confluency-induced differentiation of colon cancer cells. DLD-1, Caco-2, HT29-5M12 and HT29-5M21 cells were cultured in post-confluency for 22–30 days. Total cell lysates prepared at different time points were analysed for PMCA protein expression by Western blotting using anti-PMCA1 pAb NR1 (left panels), anti-PMCA4b mAb JA3 (middle panels) and anti-PMCA mAb 5F10 (right panels) antibodies.

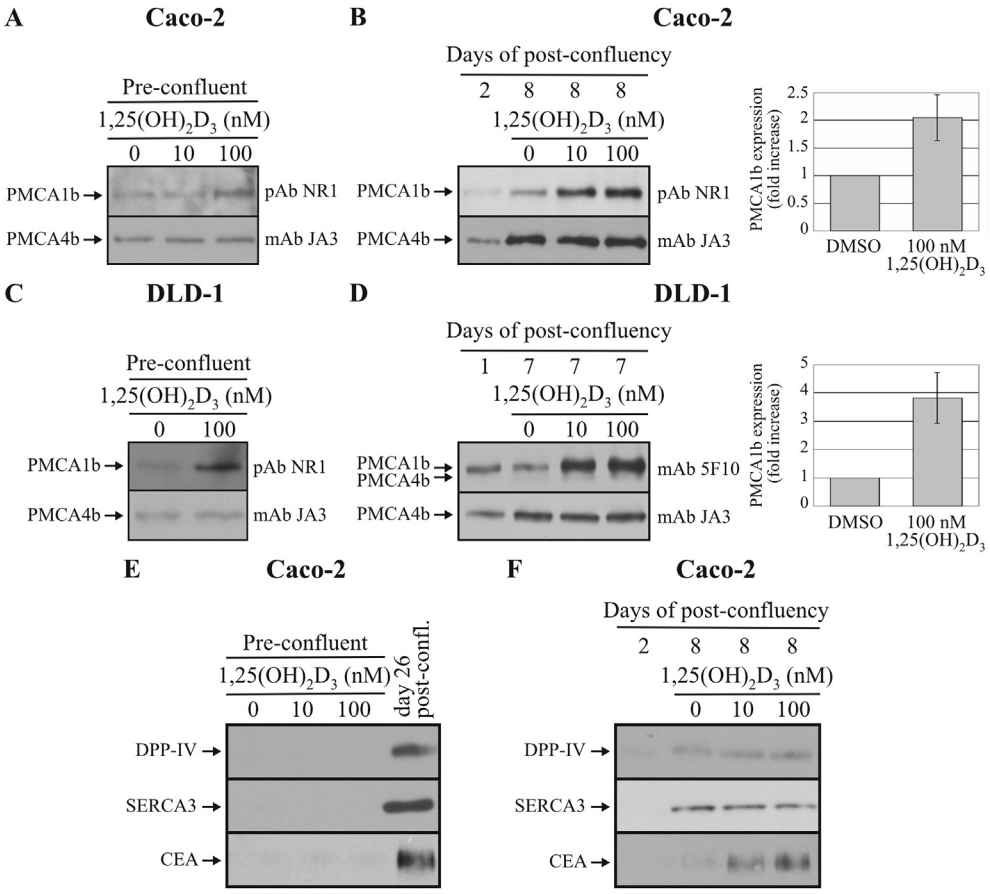

Fig. 2. Expression of PMCA isoforms and differentiation markers during $1,25(OH)_2D_3$ treatments of pre-confluent (undifferentiated) and early post-confluent (differentiating) colon cancer cells. (A–D) Pre-confluent and day 2 (Caco-2) or day 1 (DLD-1) post-confluent colon cancer cells were treated with 10 or 100 nM $1,25(OH)_2D_3$ every other day for 6 days. Total cell lysates were prepared at the indicated time points and samples were analysed for the expression of PMCA proteins by Western blotting using anti-PMCA1 pAb NR1, anti-PMCA4b mAb JA3 and the anti-PMCA mAb 5F10 antibodies. The bars represent the relative increase in PMCA1b protein expression after 6 day long 100 nM $1,25(OH)_2D_3$ treatments of day 2 (Caco-2) or day 1 (DLD-1) post-confluent cells as compared to vehicle-treated samples (means ± S.D., $n \geq 8$). (E–F) Expression of established differentiation markers (DPP-IV, SERCA3, and CEA) during 6 day long 10–100 nM $1,25(OH)_2D_3$ treatments of pre-confluent or early post-confluent Caco-2 cells, and in day 26 post-confluent Caco-2 cells.

4. Discussion

We have previously shown that in various colon cancer cell lines PMCA1b is the dominant PMCA isoform, and PMCA4b is also expressed, but at lower levels [8]. Differentiation initiated by histone deacetylase inhibitors (*e.g.,* butyrate, valerate or trichostatin A) resulted in the preferential up-regulation of PMCA4b expression [5,8].

Absorptive enterocytes and mucus-producing goblet cells are the two main epithelial cell types present in the intestinal tract. Well-characterized intestinal adenocarcinoma cell lines capable of differentiation are widely used *in vitro* models for exploring the behaviour of maturing intestinal epithelium. Therefore, we analysed the changes in the PMCA expression pattern during the post-confluent growth-induced differentiation of enterocyte-type (Caco-2, HT29-5M12, DLD-1), and goblet cell-type (HT29-5M21) intestinal adenocarcinoma model cells. Long term culture of all the tested cell lines resulted in marked induction of PMCA4b expression, whereas PMCA1b levels did not change (HT29-5M12, DLD-1, HT29-5M21), or was only moderately increased (Caco-2) (Fig. 1). We propose that the induction of PMCA4b expression is characteristic of both the absorptive enterocyte-type and the mucus-secreting goblet cell-type differentiation processes.

Dietary Ca^{2+} is absorbed mainly in the small intestine, where Ca^{2+} transport occurs *via* transcellular and paracellular pathways. $1,25(OH)_2D_3$-vitamin is a key regulator of intestinal Ca^{2+} absorption partly by controlling the expression of certain Ca^{2+} transport and Ca^{2+} binding proteins of enterocytes [15]. The regulatory effect of $1,25(OH)_2D_3$ on mammalian intestinal *PMCA1* has been previously documented in animal models, in normal endoscopic human duodenal mucosal biopsies and in Caco-2 cells at mRNA level [23–31]. However, no data were thus far available on the $1,25(OH)_2D_3$-induced regulation of PMCA1 expression in human samples at protein level. Here we show that physiologically relevant concentrations (10–100 nM) of $1,25(OH)_2D_3$ significantly and selectively up-regulate the expression of PMCA1b, but not that of PMCA4b protein in the enterocyte-type Caco-2 and DLD-1 cells (Fig. 2).

Transcellular Ca^{2+} transport accomplished by the enterocytes is composed of apical Ca^{2+} uptake, Ca^{2+} movement to the basolateral pole and Ca^{2+} extrusion through the basolateral plasma membrane. Previous data suggest that PMCA proteins have a key role in the basolateral Ca^{2+} efflux of enterocytes [32]. However, it is still not clear which PMCA isoform(s) participate in this process. As differentiated Caco-2 cells are a widely accepted *in vitro* model of small intestinal enterocytes [21,26], we analysed the subcellular localization of PMCA proteins in these cells. Furthermore, a previous study documented that $1,25(OH)_2D_3$ controls the subcellular localization of PMCA proteins in renal epithelial model cells [33]. Therefore, we also tested the potential effect of $1,25(OH)_2D_3$ on the localization of PMCA proteins in differentiated Caco-2 cells. Immunofluorescence labelling using the pan-PMCA specific 5F10,

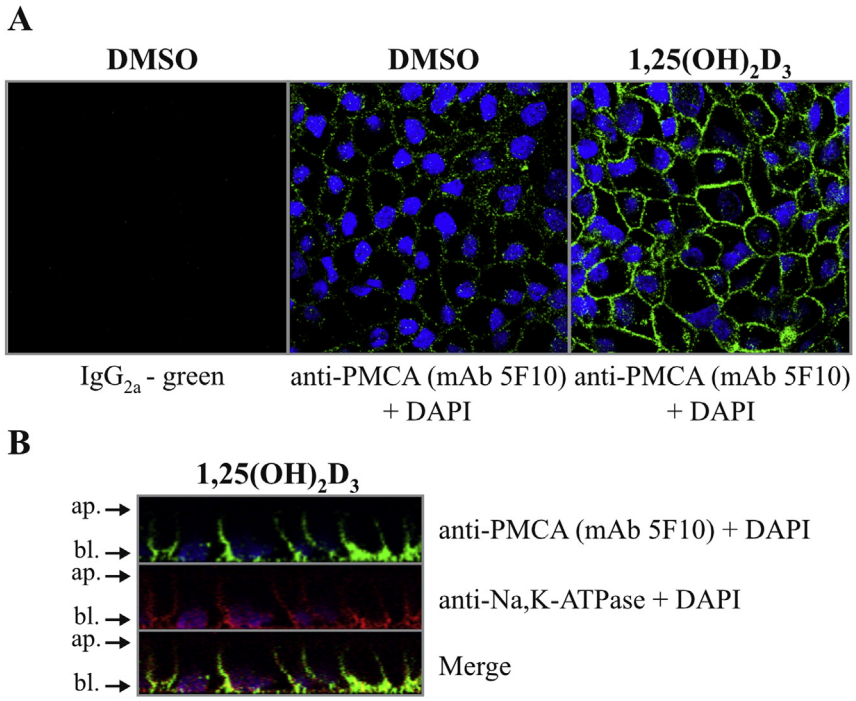

Fig. 3. Expression and localization of PMCA proteins in $1,25(OH)_2D_3$-treated differentiated Caco-2 cells. (A) Day 20 post-confluent Caco-2 cells were treated with 100 nM $1,25(OH)_2D_3$ or vehicle (DMSO) every other day for 6 days, and immunostained for PMCA with mAb 5F10 (green). Nuclei were counterstained with DAPI (blue). A DMSO-treated sample was also tested with mouse IgG_{2a} (isotype control for mAb 5F10). Images show representative x/y planes of stacks of confocal microscopy pictures. All the images were acquired using the same microscope settings. (B) Day 26 post-confluent 100 nM $1,25(OH)_2D_3$-treated Caco-2 cells were co-immunostained for PMCA (green) using mAb 5F10 and for Na,K-ATPase (red). Nuclei were counterstained with DAPI (blue). Panels show the cross sections (x/z) of stacks of confocal images. The positions of the apical and basolateral membranes are indicated as "ap."and "bl.", respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or isoform specific anti-PMCA1 and anti-PMCA4b (data not shown) antibodies demonstrated that both PMCA1b and 4b proteins are localized exclusively to the basolateral region of the plasma membrane (Fig. 3B). No difference was found in the localization of PMCA proteins in untreated *versus* 100 nM $1,25(OH)_2D_3$-treated differentiated Caco-2 cells. However, $1,25(OH)_2D_3$ greatly increased the expression of PMCA proteins in differentiated Caco-2 cells (Fig. 3A). Based on the Western blot analysis (Fig. 2B), this is presumably due to the elevation in total PMCA1b expression. We propose that $1,25(OH)_2D_3$ does not control the localization, but regulates the expression of PMCA pumps in intestinal enterocytes. As both the PMCA1b and 4b proteins localize to the basolateral plasma membrane, we suppose that both isoforms participate in the basolateral Ca^{2+} efflux of enterocytes. The selective regulatory effect of $1,25(OH)_2D_3$ on the expression of PMCA1b, however, further suggests that PMCA1b plays a preferential role in $1,25(OH)_2D_3$-induced intestinal Ca^{2+} absorption.

Accumulating epidemiological and nutritional data indicate that $1,25(OH)_2D_3$ is protective against the malignancies of the intestinal tract [16,17]. However, the anti-tumour activities of $1,25(OH)_2D_3$ on intestinal adenocarcinoma cells are less known. Here we tested the differentiation inducing/potentiating activity of $1,25(OH)_2D_3$ on Caco-2 cells by monitoring the expression of well-established differentiation markers (DPP-IV, CEA, SERCA3). 10–100 nM $1,25(OH)_2D_3$ treatments had no effect on the expression of the studied differentiation markers in preconfluent Caco-2 cells (Fig. 2E) and only increased the expression of CEA in early post-confluent differentiating Caco-2 cells (Fig. 2F). These results lead to the hypothesis that $1,25(OH)_2D_3$ does not control the global differentiation process, but it can regulate the expression of certain differentiation-related genes in colon cancer cells.

In summary, data presented in this work show that the regulation of the expression of various PMCA isoforms, co-expressed in the same cell, is associated with distinct physiological programs/functions. Although PMCA1b and 4b enzymes accomplish a shared basic function (*i.e.,* Ca^{2+} extrusion from the cell), our data indicate that whereas PMCA4b expression levels vary in function of cell differentiation along both the absorptive enterocytic and the goblet cell phenotype, the expression of PMCA1b is relatively insensitive to differentiation *per se*. On the other hand, PMCA1b expression is selectively sensitive to the effect of $1,25(OH)_2D_3$, an agent involved in the enhancement of intestinal Ca^{2+} uptake. We suggest that PMCA1b may be particularly well suited to perform house-keeping Ca^{2+} extrusion through the basolateral plasma membrane, leading to Ca^{2+} absorption in the organism, a mechanism highly regulated by $1,25(OH)_2D_3$. Our work illustrates that the accomplishment of specific functions by various, functionally slightly different PMCA isoforms of shared overall biochemical activity constitutes a previously unrecognized level of specialization of cellular Ca^{2+} transport and homeostasis.

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