Functional and Structural Demonstration of the Presence of Ca-ATPase (PMCA) in Both Microvillous and Basal Plasma Membranes from Syncytiotrophoblast of Human Term Placenta

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Abstract

It is known that human syncytiotrophoblast (hSCT) actively transports more than 80% of the Ca^{2+} that goes from maternal to fetal circulation. Transepithelial transport of Ca^{2+} is carried out through channels, transporters and exchangers located in both microvillous (MVM) and basal (BM) plasma membranes. The plasma membrane Ca-ATPase (PMCA) is the most important mechanism of Ca^{2+} homeostasis control in the human placenta. In this work, we reexamined the distribution of PMCA in isolated hSCT of term placenta. The PMCA activity was determined in isolated hSCT plasma membranes. A partial characterization of the PMCA activity was performed, including an evaluation of the sensitivity of this enzyme to an *in vitro* induced lipid peroxidation. Expression of the PMCA in hSCT plasma membranes and tissue sections was investigated using Western blots and immunohistochemistry, respectively. Our study demonstrates, for the first time, a correlation between the activity and structural distribution of PMCA in both MVM and BM of hSCT. It also demonstrates a higher PMCA activity and expression in MVM as compared to BM. Finally, PMCA4 seems to be preferentially distributed in both hSCT plasma membranes, while PMCA1 is shown to be present in the hSCT homogenate. However, the membrane fractions did not show any PMCA1 labeling. Our results must be taken into account in order to propose a new model for the transport of calcium across the hSCT.

Keywords: PMCA; Placenta; Syncytiotrophoblast; Microvillous plasma membranes; Basal plasma membranes

1. Introduction

It is known that Ca^{2+} plays a key role in intracellular signaling in many cells. As a consequence, the intracellular Ca^{2+} concentration should be tightly regulated [1]. This control is exerted through various mechanisms, among which the plasma membrane Ca-ATPase (PMCA) plays an important role in the maintenance of low free Ca^{2+} concentration in the cytoplasm, by extruding cytosolic Ca^{2+} from the cells [2]. This enzyme is known to be coded by four separate genes (PMCA 1−4), and due to alternative splicing, there are at least 20 variants designated by lowercase letters following the isoform number (e.g., $PMCA1_a$, $PMCA1_b$, etc.) [3,4]. PMCA 2 and 3 isoforms are found in specialized tissues while isoforms 1 and 4 are present in almost all tissues including placenta [5]. However, there is a high variability in the patterns of expression for the different splice variants of the PMCA isoforms. For instance, in placental tissues only $PMCA4_a$, $PMCA4_b$ and $PMCA1_a$ are present [6]. The different PMCA isoforms differ primarily in their regulatory regions and the modulation

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of their activities strongly depends on the membrane composition [3,4].

The human placental syncytiotrophoblast (hSCT) is a polarized epithelium, representing the interface between the maternal and fetal circulations. This epithelium has a maternal-facing microvillous membrane (MVM) and a fetal-facing basal membrane (BM). Thus, any transport of ions and solutes to and from the fetal compartment must be performed across both MVM and BM. In particular, it is known that the syncytiotrophoblast actively transport more than 80% of the Ca^{2+} that goes from maternal to fetal circulation [7]. Transepithelial transport of Ca^{2+} is carried out by channels, transporters and exchangers located in both MVM and BM [8].

Placental Ca^{2+} transport has been reported to be inhibited by the addition of erythrosin B, a placental PMCA inhibitor [9]. In addition, Ca^{2+} transport in perfusion studies of placenta, is minimally affected when the Na^{+} of the incubation medium is substituted with choline, a clear indication that the Na^{+}/Ca^{2+} exchanger (NCX) has a minimal role in the transplacental movement of Ca^{2+} from mother to fetus [10]. Accordingly, it has been proposed that PMCA plays an important role in Ca^{2+} homeostasis in the human placenta [11]. One of the very first studies looking for PMCA in hSCT, considered the fact of a higher concentration of calcium in the fetal circulation as compared to the maternal one, suggesting a predominant presence of such an extrusion mechanism in BM [12]. This consideration led these authors to demonstrate the presence of PMCA in membrane vesicles of BM from hSCT, without paying attention to MVM. In another set of studies, Borke et al. [13] and Strid and Powell [14], by using immunohistochemistry techniques in human placental tissue and with a monoclonal anti-PMCA antibody 5F10, found an apparent exclusive distribution of PMCA in BM. In addition, Borke et al. [13], determined from the Western blot analysis of MVM preparations that the antibody 5F10 binds poorly to the region around 140 kDa. These specific findings have been used, since then, to conclude that the PMCA, similarly to renal and intestinal epithelia, is located exclusively in BM from hSCT [8]. However, in preliminary experiments we have found: (a) the presence of a Mg^{2+}-dependent, thapsigargin-insensitive, Ca^{2+}-stimulated-ATPase activity in MVM fractions isolated from human term placenta [15]; and (b) PMCA expression in MVM fractions of placental membranes by Western blot and immunohistochemistry with specific antibodies [16,17]. These findings, together with the fact that the PMCA and the Na,K-ATPase (NKA) are colocalized in the basolateral plasma membranes of renal and intestinal epithelia, while the NKA is present in both BM and MVM from hSCT [18], led us to reexamine the distribution of PMCA in hSCT of term placenta. In this study, we tested the hypothesis that PMCA is present in both MVM and BM from hSCT. The biochemical expression of the PMCA activity was assayed and partially characterized in isolated preparations from both MVM and BM. Expression of the PMCA in isolated hSCT plasma membranes and tissue sections was also investigated using Western blots and immunohistochemistry, respectively.

2. Materials and methods

2.1. Placenta collection

Placentae obtained from normal pregnancies were collected immediately after delivery from the Maternity Hospital "Concepción Palacios" in Caracas, Venezuela and San José Hospital Maternity Unit (monitored Unit Outpatient clinic, healthy pregnancies) in Santiago, Chile and transported to the respective laboratories on ice. Any woman that, according to her medical history, was under medical treatment to control blood pressure, or if she was taking >1 g of elemental calcium per day during pregnancy, or if she had a history of hypertension, diabetes, calcium metabolism disorders, or any other chronic medical illness, was not considered for this study.

2.2. Preparation of syncytiotrophoblast plasma membranes

The human placental plasma membranes (MVM) and basal membranes (BM) were prepared from fresh placentae following a previously described method [19,20]. In brief: the maternal decidua was removed, and the central portion between the maternal and fetal surfaces was used for the preparation. Placental villous tissue (80−100 g) was chopped into small pieces, washed with 0.9% NaCl to remove blood and filtered through gauze. The purification method involved different steps: differential centrifugation, precipitation of non-microvillous membranes with magnesium ions and a sucrose gradient step. All solutions were buffered with 20 mM Tris-maleate, pH 7.4. Sucrose gradient preparation: a portion (2−3 ml) of the microvillous-enriched preparation and the basal membrane-enriched preparation were overlayed on the sucrose gradient. The band at the sucrose interface concentrations 37/45% (w/v) corresponds to the apical fraction (MVM) and the band at the sucrose interface concentrations 47/52% (w/v) corresponds to the basal fraction (BM). The two fractions were collected, diluted 10-fold with the buffer 20 mM Tris-maleate, pH 7.4, and centrifuged at 110,000 × g for 30 min. The final pellets were resuspended in 300 mM sucrose, 20 mM Tris-maleate, pH 7.4, and stored in liquid nitrogen or at −50 °C (freezer). The purity and enrichment of the membrane fractions were determined routinely by assaying alkaline phosphatase, an apical membrane marker; adenylate cyclase/β-adrenergic receptor (by measuring ^{3}H-dihydroalprenolol binding), basal membrane markers; cytochrome c oxidase/succinate dehydrogenase, mitochondrial membrane markers and glucose-6-phosphatase, endoplasmic reticulum marker [12,20].

2.3. PMCA activity

The PMCA activity was determined by measuring the quantity of inorganic phosphate liberated from the hydrolysis of ATP, according to a modification of the method described elsewhere [21]. Briefly, 180 µl of the incubation medium were preincubated for 2 min at 37 °C, and the reaction was started by addition of 20 µl of membrane suspension. After 10 min incubation, the reaction was stopped by addition of 300 µl of a cold solution containing: 2.85% ascorbic acid; 1.76% HCl; 0.48% ammonium molybdate; and 2.85% SDS. The samples were shaken and kept at 0 °C for 10 min. Then, 500 µl of 2% sodium citrate, 2% sodium arsenite and 2% glacial acetic acid solution were added to each tube, which were then rewarmed, after shaking, for 10 min at 37 °C. The absorbance of each tube was determined in a Milton Roy spectrophotometer at 705 nm. The ATPase activity is expressed as nmol Pi/mg protein min, after subtraction of a blank run in parallel under the same conditions except for the membrane suspension, which was added only after the addition of the ascorbic acid solution. The protein concentration, in all the cases, was determined according to the method of Bradford [22]. The PMCA activity was calculated as the difference in the phosphate liberated in a medium containing 250 mM sucrose; 5 mM ATP; 5 mM $MgCl_2$; 1 mM ouabain; 2 mM EGTA; 2 mM EDTA; 30 mM Tris−HCl (pH 7.2 at 37 °C); 55 mM KCl, 2 µg/ml calmodulin; 1 µM thapsigargin and 2 µM free calcium, minus the one liberated in the same medium, but in the absence of calcium. Purified bovine brain calmodulin was generously supplied by Dr. Gustavo Benaim from the Universidad Central de Venezuela.

2.4. SDS pretreatment of the syncytiotrophoblast plasma membranes

In order to avoid the presence of membrane vesicles, the membrane fractions were always pretreated before the assays with SDS, as previously described [23]. A 250 µl aliquot of the fraction (0.4 mg protein/ml) was pretreated with up to 8 µl of a solution containing 6.25 µg SDS/µl, 1% BSA, 25 mM Imidazole pH 7.2 at 37 °C. The fractions were incubated for 20 min at 37 °C, and then immediately assayed for ATPase activity.

2.5. Ultraviolet irradiation of the syncytiotrophoblast plasma membranes

A 750 µl aliquot of the syncytiotrophoblast plasma membranes (1 mg protein/ml) from normal pregnant women was poured into a glass vial, placed on ice, and illuminated from approximately 4 cm distance by a mineral light (wavelength 254 nm maximum, specified strength 280 µW/cm^2 at 15 cm distance) for different periods of time to induce lipid peroxidation [24]. The UV irradiation of the hSCT plasma membranes was carried out in the presence and absence of 50 µM butylated hydroxytoluene (BHT).

2.6. Lipid peroxidation measurements

The amount of lipid peroxidation of the plasma membranes was estimated by measuring the thiobarbituric acid-reactive substances (TBARS). The TBARS were determined according to the method described by Feix et al. [25]. The absorbance was measured at 532 nm and the TBARS values were calculated by using a malondialdehyde standard curve, prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane. The values are expressed as nmoles of malondialdehyde per milligram of protein.

2.7. Immunohistochemistry

Human placental tissue samples (0.5 cm^3) from normal pregnancies were placed in 0.9% NaCl and fixed in 3.7% buffered formaline at pH 7.4, for 24 h minimum. Subsequently, the tissue was rinsed five times in ice-cold phosphate buffered saline (PBS) and dehydrated through a graded series of ethanol to xylene, embedded in paraffin, and cut into 5 µm-thick sections. Afterwards, paraffin was removed in xylene and the sections were rehydrated by passage through graded ethanol and, finally, distilled water. The sections were blocked for 1 h at room temperature with 4% bovine serum albumin (BSA) in PBS. Tissue was then incubated for 2 h at room temperature with a monoclonal antibody raised against all isoforms of PMCA (clone 5F10) diluted 1:1000, a monoclonal antibody against PMCA4 isoform diluted 1:1000 (clone JA9); a rabbit polyclonal antibody against PMCA1 isoform diluted 1:500 (all from Affinity Bioreagents, CO) and a mouse monoclonal antibody against human placental alkaline phosphatase (anti-PLAP, clone 8B6; Sigma-Aldrich, Inc.) in 2% BSA in PBS. Negative control sections were treated similarly, except that primary antibodies were omitted. After rinsing the samples with PBS, tissue sections were incubated for 1 h at room temperature with biotin conjugated goat anti-mouse and biotin conjugated goat anti-rabbit antibodies, for monoclonal and polyclonal primary antibodies used respectively (Vector Laboratories, Burlingame, CA). The secondary antibody was rinsed off with three changes of PBS, and Horseradish peroxidase-streptavidin reagent was added for 30 min at room temperature. Slides were finally treated with NOVAREDÒ (Vector Laboratories, Burlingame, CA) to visualize the antigen stain. After the appearance of a red reaction product, slides were washed in PBS, dehydrated in graded ethanol, cleared in xylene, and mounted in ENTELLAN® (Merck). Before dehydration, the slides were counterstained using hematoxylin.

2.8. Electrophoresis, Western blotting and densitometric analysis

Aliquots of homogenate (H), MVM and BM preparations (20 µg total protein) were separated on a 7.5% SDS-PAGE gel. Electrophoresis was performed at 100 V and the gel was transferred to a nitrocellulose membrane (BioRad 162-0115) for 2 h at 100 V. The nitrocellulose membrane was blocked for 2 h at room temperature with 3% non-fat milk in saline buffer-Tween (138 mM NaCl, 270 mM KCl and 0.05% Tween 20), and washed in saline buffer-Tween. The membranes were incubated with a primary antibody for 2 h at room temperature, i.e. anti-PMCA monoclonal antibody (clone 5F10, diluted 1:1000), anti-PMCA4 monoclonal antibody (clone JA9, diluted 1:1000) and rabbit polyclonal antibody against PMCA1 isoform, diluted 1:500 (all from Affinity Bioreagents, CO). After washing with saline buffer-Tween, the membranes were incubated with the specific secondary antibody; anti-mouse horseradish peroxidase-linked antibody (1:10,000), and incubated for 1 h at room temperature. The final detection was done using the enhanced chemiluminiscence Western blotting analysis system (ECL, Amersham, RPN 2106). Protein content was quantified using UN-SCAN-IT gel Automated Digitizing System, version 4.1 (Silk Scientific Corporation).

2.9. Statistical analysis

Comparisons between the different conditions were assessed by one-way ANOVA followed by post hoc analysis with the Student-Newman−Keuls test. All results are expressed as means ± SE and (n) represents the number of experiments performed with different preparations. The PMCA activity was calculated from paired data. A p-value ≤ 0.05 was accepted as statistically significant.

3. Results

3.1. Purity of MVM and BM fractions

The plasma membrane preparations of hSCT were carefully evaluated for membrane purity and cross contamination. Table 1 shows the enrichment factors, calculated as the ratio of activity in membrane fractions to that in the homogenate (H), of the different membrane markers. The MVM fraction showed an enrichment factor of alkaline phosphatase (PLAP) of around 21-fold, and low contamination with BM, as indicated by the enrichment factor of the BM markers (adenylate cyclase activity and ^{3}H-dihydroalprenolol binding). The BM fraction also showed an important enrichment, as judged by adenylate cyclase activity (around 12-fold when compared to the activity in the microsomal fraction) and ^{3}H-dihydroalprenolol binding (around 13-fold). Cross contamination of the BM fraction with MVM was low, as indicated by the 2-fold enrichment of PLAP in the BM fraction. The ratio between the adenylate cyclase activity of MVM and BM fractions (MVM/BM) was 0.09, indicating a very low

Table 1
Comparison of the enrichment factor of several membrane markers in basal (BM) and microvillous (MVM) plasma membrane fractions from human term placenta

Marker	BM	MVM
Alkaline phosphatase	2.31 ± 0.37	21.90 ± 3.65
^{3}H-dihydroalprenolol binding	13.20 ± 0.61	2.23 ± 0.22
Adenylate cyclase*	12.15 ± 0.20	0.71 ± 0.11
Succinate dehydrogenase	1.63 ± 0.30	2.80 ± 0.44
Glucose-6-phosphatase	0.23 ± 0.02	1.09 ± 0.07

The enrichment factors were calculated as the ratio of activity in membrane fractions to that in the homogenate (H). Values are means ± SE of eight determinations carried out with different preparations. *The enrichment factor was calculated versus the activity of the microsomal fraction from our preparations.

contamination of MVM by BM. Similarly, the same ratio, calculated for the ^{3}H-dihydroalprenolol binding was also very low: 0.20 (data not shown). Both BM and MVM fractions showed a poor contamination with mitochondrial membranes and endoplasmic reticulum. The purity and cross contamination of the purified membranes were comparable to those reported for single or paired apical and basal membrane preparations [19,20].

3.2. PMCA activity of MVM and BM fractions

Both membrane preparations (MVM and BM) were assayed for PMCA activity. The biochemical criteria used to identify the PMCA activity included the determination of an Mg^{2+}-dependent ATPase activity, stimulated by free Ca^{2+} in the micromolar range and by calmodulin, a well-known modulator of this ATPase activity [2]. PMCA activity is inhibited by 30 μM vanadate (a potent inhibitor of P-type ATPases such as PMCA [2]) and is insensitive to 2 mM ouabain (Na,K-ATPase inhibitor) and 1 μM thapsigargin (SERCA inhibitor). Before the ATPase assays, the membrane preparations were pretreated with SDS in order to avoid the presence of membrane vesicles [23]. The results of the pretreatment with SDS of both BM and MVM on the PMCA activity are shown in Fig. 1. For both membrane preparations, the maximal ATPase activity was reached when the membranes were pretreated with 0.25 μg SDS/μg protein. Consequently, before any PMCA assay, the membranes were always pretreated with this SDS/protein ratio.

Table 2 shows the PMCA activity in H, MVM and BM fractions. It can be seen that the PMCA activity is not only present in both MVM and BM, but also that the activity in MVM is about twice as much as that in BM.

3.3. Partial characterization of PMCA activity in hSCT plasma membranes

The PMCA activity in MVM and BM preparations was further analyzed. A partial characterization of the activity of both enzymes is presented in Table 3. The different parameters analyzed were almost identical for both membrane preparations, which showed similar K_m for free Ca^{2+} stimulation, similar calmodulin modulation, similar optimal pH and temperature values of the assay medium, and insensitivity to 2 mM ouabain and 1 μM thapsigargin. In addition, we tested the effect of 30 μM vanadate, a P-type ATPase inhibitor, on the PMCA activity of both MVM and BM. At the indicated concentration, vanadate produced almost complete inhibition of the PMCA in both membrane fractions (Table 3), indicating that these ATPase activities correspond to the P-type ATPases.

Considering the fact that the PMCA activity of human red cell ghosts is quite sensitive to membrane lipid peroxidation [26], we utilized this parameter to further characterize the PMCA activity in MVM and BM. Lipid peroxidation was induced by UV light irradiation at 4 °C for different lengths of time, and determination of TBARS was used as an indication of the level of lipid peroxidation of the membranes [25].

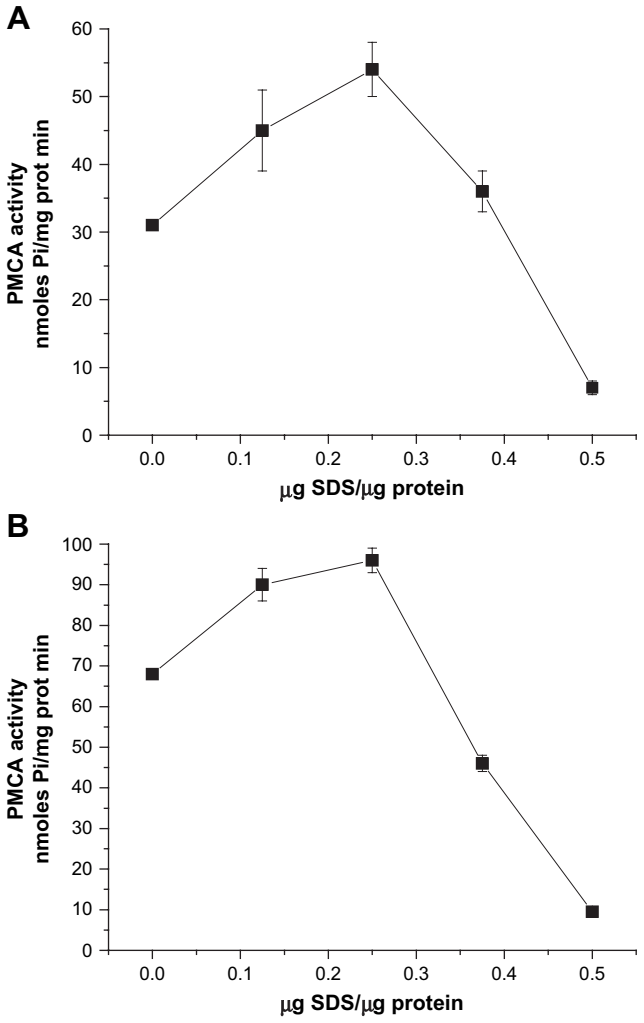

Fig. 1. Effect of the treatment of BM (panel A) and MVM (panel B) from hSCT with SDS on the PMCA activity. Values are expressed as means ± SE of six determinations carried out with different preparations.

The treatment was carried out in the presence and absence of the antioxidant butylated hydroxytoluene (BHT). As shown in Fig. 2, for both preparations, the TBARS of the plasma membranes increased proportionally as a function of the UV light treatment time, while the PMCA activity decreased proportionally to this increment. When the UV irradiation of the hSCT plasma membranes of both samples was carried out in the presence of 50 μM BHT, the two measured parameters PMCA activity and level of lipid peroxidation, remained unaffected (data not shown). This is a clear indication that the UV

Table 2
PMCA activity of the microvillous (MVM) and basal (BM) plasma membrane fractions of human term placenta

Fraction	ATPase activity: nmoles Pi/mg prot min
H	5 ± 2
BM	51 ± 5
MVM	92 ± 4

Values are means ± SE of five determinations carried out with different preparations.

Table 3
Partial characterization of the PMCA activity of microvillous (MVM) and basal (BM) plasma membrane fractions of human term placenta

Parameter	BM	MVM
K_m for free Ca^{2+} (μmol/l)	0.22 ± 0.03*	0.25 ± 0.02*
Calmodulin stimulation	Yes	Yes
Optimal pH	7.4	7.4
Optimal temperature	42 °C	42 °C
% inhibition by vanadate (30 μM)	95 ± 2*	97 ± 3*
Ouabain sensitivity (2 mM)	None	None
Thapsigargin sensitivity (1 μM)	None	None

*Values are means ± SE of six determinations carried out with different preparations.

treatment of the plasma membranes, *per se*, does not affect the structure of membrane proteins, such as the PMCA. Data from Fig. 2 were utilized to perform a regression analysis of the PMCA activity as a function of the TBARS, for both MVM and BM. This plot is shown in Fig. 3. Although the sensitivity of the PMCA activity to lipid peroxidation is different for both membranes, as indicated by the different slopes of the regression lines, it is evident that in both cases, the PMCA activity is quite sensitive to the level of lipid peroxidation of the plasma membrane (coefficient of determination, $r^2 = 0.924$ and 0.945 for BM and MVM, respectively).

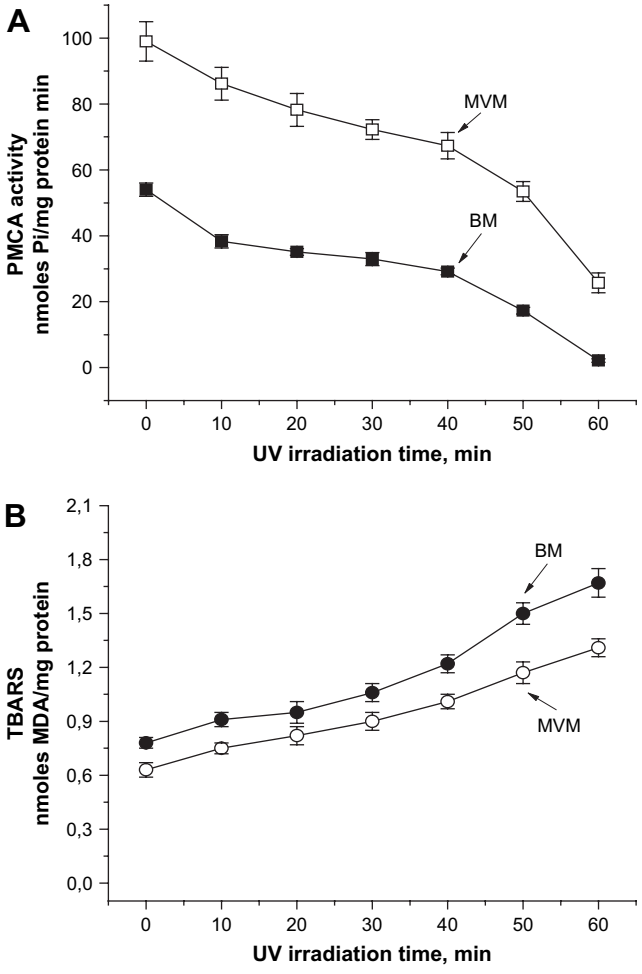

Fig. 2. PMCA activity (panel A) and TBARS (panel B) as a function of the irradiation time with UV light (254 nm) of both BM (filled symbols) and MVM (empty symbols) from hSCT. Values are expressed as means ± SE of six determinations carried out with different preparations.

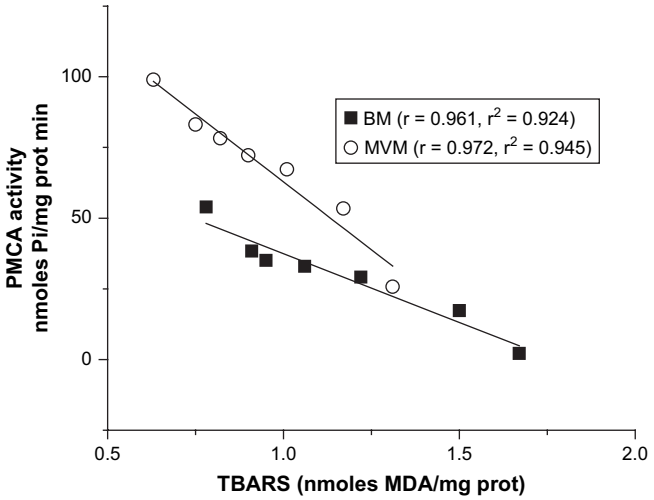

Fig. 3. PMCA activity as a function of the level of lipid peroxidation (TBARS) of BM and MVM from hSCT. Data were taken from the mean values of Fig. 2.

3.4. Expression of PMCA in plasma membranes from human syncytiotrophoblast

The Western blot analysis of the membrane fractions was carried out with a monoclonal antibody against all isoforms of PMCA (clone 5F10), a monoclonal against PMCA4 (clone JA9) and a polyclonal antibody against PMCA1 isoform. The antibody 5F10 detects the four PMCA isoforms, including their respective splice variants, because it recognizes an epitope that is common to all known human PMCA gene products and splicing variants [27]. Western blot analyses were performed in samples of paired H, MVM and BM purified fractions from a total of six placentae. Fig. 4A shows a representative Western blot (inset) using the antibody 5F10. It can be seen that the preparations show a main band around 140 kDa, which is in agreement with the molecular weight of the PMCA [2]. The second band, which co-migrated with the 140 kDa band, has been previously seen in red cell ghosts and BM from hSCT preparations [28–30], and it has been suggested to be a splice variant of the active PMCA. As shown by the relative density, the expression of PMCA is higher in MVM than in BM. The basal PMCA band corresponds to less than 20% of the sum of apical and basal PMCA densitometrically quantified bands. Comparable results were obtained by using a monoclonal antibody directed towards PMCA4, as shown in Fig. 4B. The Western blot analyses confirm our data of PMCA activity (Table 2), showing the presence of this enzyme in both MVM and BM, with higher ATPase activity in MVM.

Additional experiments were performed in order to try to identify the presence of the PMCA1 isoform in the hSCT plasma membranes. When a rabbit polyclonal antibody against PMCA1 isoform was used, there was a strong mark in H (Fig. 4C); however, none of the purified fractions showed a specific band for PMCA1.

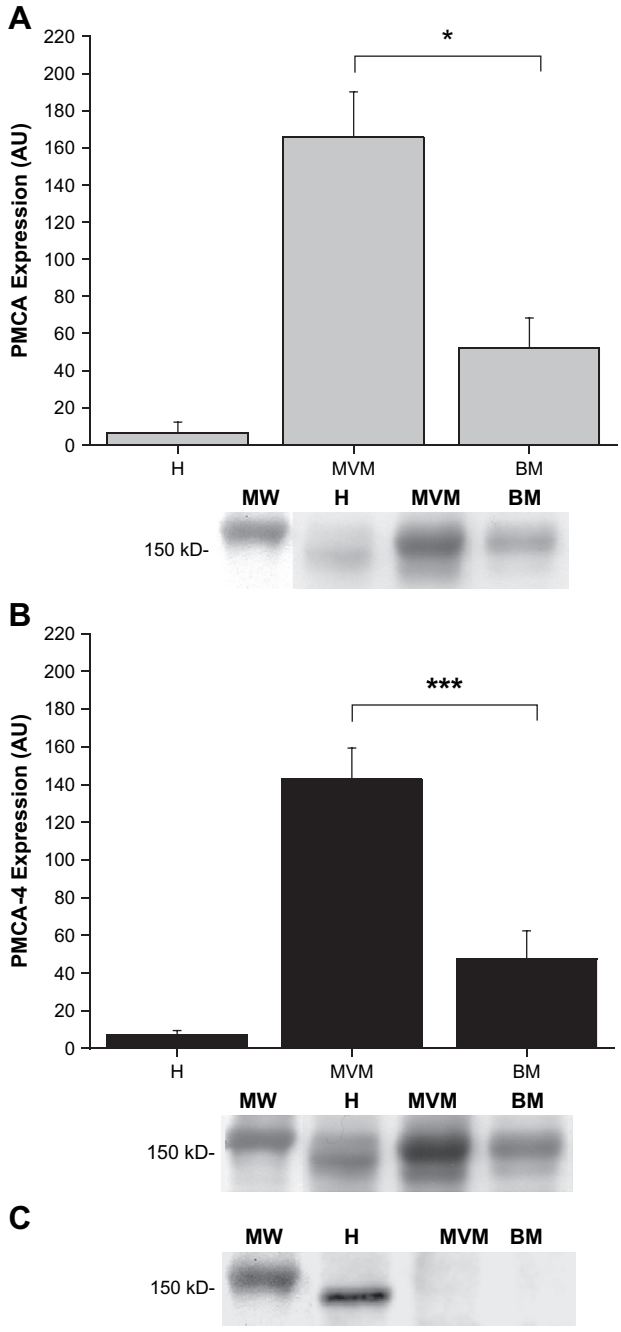

Fig. 4. Western blotting and densitometric analysis for PMCA and PMCA 4 isoform in MVM and BM syncytiotrophoblast purified membranes. (A) Histograms of Western blot analysis and representative image (inset) show that total PMCA expression is observed in both membranes and that the PMCA mark is bigger in MVM, as compared to the BM fraction. (B) Histograms of Western blot analysis and representative image (inset) for PMCA4 isoform. (C) Western blot for PMCA1 isoform. The histograms summarize quantification of densitometric analyses from six independent placentae, expressed as Arbitrary Units (AU) (*$p < 0.05$; ***$p < 0.005$).

3.5. Immunohistochemistry of placental villous tissue

Further experiments were performed in order to try to identify the location of PMCA in placental villous tissue. The tissue sections (5 μm) were counterstained with hematoxylin to mark the syncytiotrophoblast nucleus. To control the antigenicity and intactness of the placental epithelium, the presence of PLAP, an epithelial apical membrane marker, was confirmed. Fig. 5B shows an immunohistochemical staining of placental villous tissue section from a normal pregnancy, with anti-human PLAP (clone 8B6; Sigma-Aldrich, Inc). It can be observed that this marker is immunolocalized in the maternal-facing side of the hSCT, which is in agreement with previous reports [19,20], and with our biochemical results (Table 1). Subsequently, monoclonal antibodies against all PMCA isoforms (clone 5F10), against PMCA4 isoform (clone JA9) and against PMCA1 isoform were used in paraffin-embedded sections of human placental villous tissue. As shown in Fig. 5C,D, PMCA staining for all isoforms was predominantly seen in hSCT, with partial expression in the fetal vascular endothelium. The syncytiotrophoblast showed specific staining for PMCA of the fetal-facing and maternal-facing sides, including a trophoblast mark. Similar distribution for PMCA4 isoform was also observed (Fig. 5E,F) but apparently weaker in the cytoplasm. In contrast, the staining of PMCA1 isoform (Fig. 5H), seems to be mainly in the trophoblast in all the studied samples. The latter observations are in agreement with the Western blot results mentioned above (Fig. 4C), where there was only evidence of the PMCA1 isoform in the placental homogenate and no staining was obtained in the purified hSCT plasma membranes. Negative control sections, where primary antibodies were omitted, showed no specific staining for the different monoclonal and polyclonal antibodies used (Fig. 5A,G, respectively). Similar results were obtained in four placentae.

4. Discussion

This study describes, for the first time, a correlation between activity and structural distribution of the plasma membrane Ca-ATPase (PMCA) in both, MVM (maternal-facing) and BM (fetal-facing) plasma membranes from the syncytiotrophoblast of human placenta. It also demonstrates higher PMCA activity and expression in MVM as compared to BM. Since the percentage of cross contamination of BM in the MVM fraction is very low (Table 1) the probability that the high expression of PMCA in MVM could be due to the presence of BM in the MVM fractions seems to be very unlikely. Specific PMCA activity was identified in both MVM and BM membranes (Tables 2 and 3), which, similar to red blood cell ghosts [26], is quite sensitive to the level of lipid peroxidation of the membranes (Figs. 2 and 3). PMCA activity of both MVM and BM, show a similar behavior for the tested biochemical parameters, i.e. K_m for free Ca^{2+}; calmodulin stimulation; optimal pH and temperature values; vanadate, ouabain and thapsigargin sensitivities (Table 3) as well as its sensitivity to UV-induced lipid peroxidation (Figs. 2 and 3).

The immunohistochemistry results confirm the presence of PMCA in polarized plasma membranes of hSCT. Interestingly, both the PMCA activity (Table 2) and the Western blot data (Fig. 4) indicate that the PMCA in MVM is, at least, twice as much as that in BM, probably due to a higher PMCA

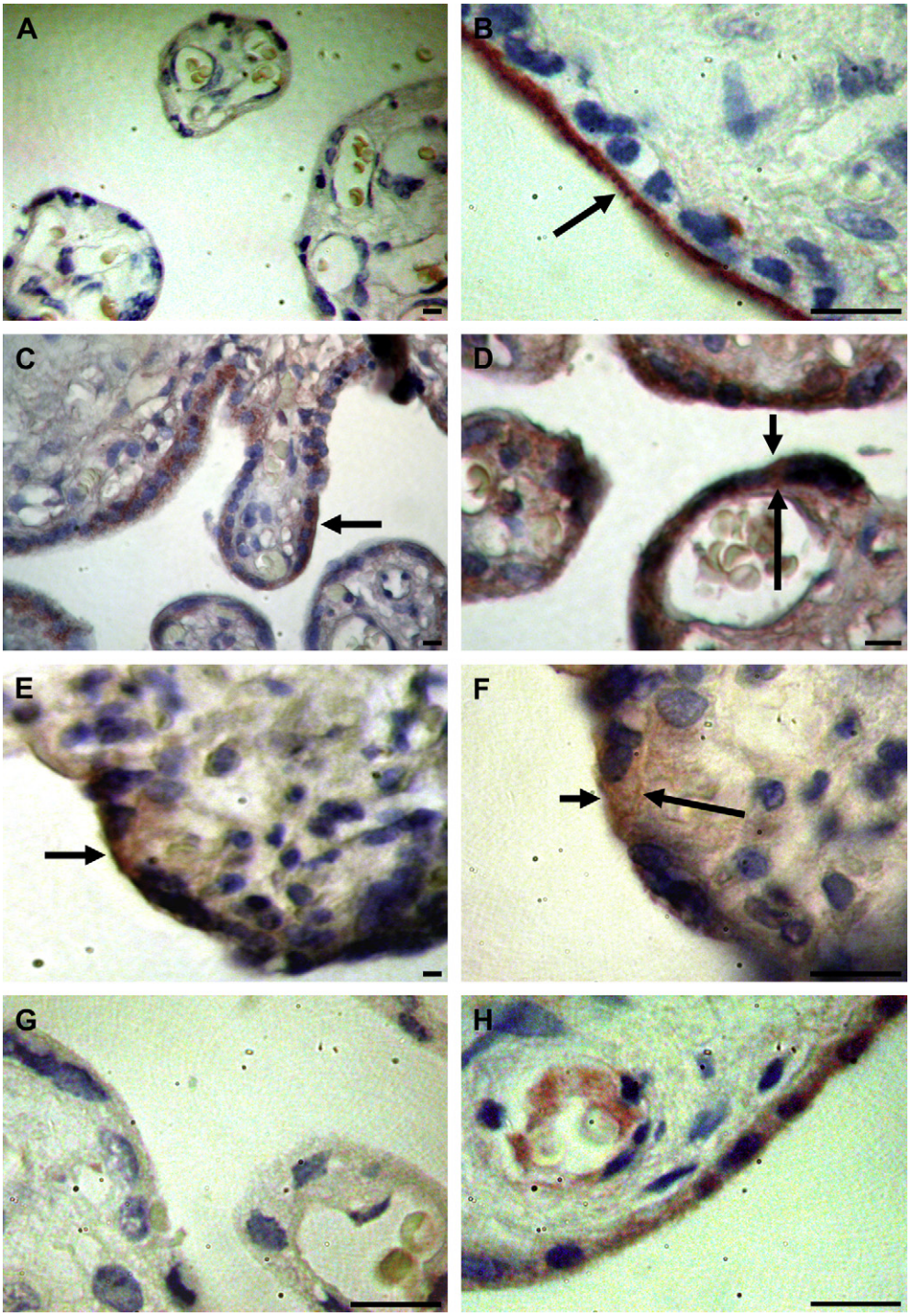

Fig. 5. Immunohistochemical localization of PMCA in human placental tissue. Panels A through H are placental villous sections showing the hSCT. Scale bar for all panels represents 10 μm. (A) Control tissue using only secondary goat anti-mouse antibody, without primary antibody (40×). (B) Tissue section stained with antibody against PLAP (clone 8B6). The arrow indicates MVM specific staining (100×). (C) Immunostaining of tissue section with antibody against all PMCA isoforms (clone 5F10). The arrow indicates specific PMCA staining, predominantly in hSCT (40×). (D) Detailed microphotography. PMCA immunostaining was located in the microvillous (short arrow) and basal (long arrow) membranes and it was also distributed in hSCT cell cytoplasm. (E) Immunostaining with antibody against PMCA4 isoform (clone JA9) revealed specific staining similar to that obtained with antibody against all PMCA isoforms (arrow, 40×). (F) Detailed microphotography: PMCA4 is located in the microvillous (short arrow) and in the basal (long arrow) hSCT membranes (100×). (G) Negative control was performed by omitting primary and using only secondary goat anti-rabbit antibody (100×). (H) Immunostaining with antibody against PMCA1 isoform. PMCA1 staining is distributed in hSCT cell cytoplasm (100×). Nuclei in blue were hematoxylin-stained.

density in MVM. It is clear that, similar to the NKA in hSCT [18] and in liver canaliculi epithelial cells [31], the distribution of PMCA in hSCT does not follow the pattern of strict polarization of this ATPase seen in classical transporting epithelia. It is not localized only in the basal membrane, but in both MVM and BM.

It is known that in placental tissues there are only $PMCA4_a$, $PMCA4_b$ and $PMCA1_a$ isoforms [6]. On this regard, our results are in agreement with these findings. The antibody JA9 reacts specifically against both $PMCA4_a$ and $PMCA4_b$ variants. Fig. 4B seems to show a doublet band (presumably 129 and 134 kDa) in all samples indicating the presence of both $PMCA4_a$ and $PMCA4_b$ variants. On the other hand, the presence of the PMCA1 isoform in the hSCT plasma membranes was detected with Western blotting of the homogenates; however, none of the purified fractions showed a specific band for PMCA1 (Fig. 4C). It is clear that this point deserves further studies in order to identify the possible role of PMCA1 in hSCT.

The cholesterol content and the phospholipids saturation index of MVM are known to be higher than those of BM [32], indicating a lower fluidity in the former. Accordingly, since both PMCA and NKA activities are sensitive to high cholesterol content and low fluidity of the plasma membranes [33,34], it could be expected a lower activity of both enzymes in MVM. However, PMCA and NKA show higher activity values in MVM than in BM (ref. [15] and Table 2). This could be interpreted as an indication of either a higher density of these ATPase molecules in MVM than in BM, which is in agreement with our Western blots results (ref. [15] and Fig. 4), or a differential sensitivity of these ATPases to both cholesterol/phospholipids ratio and fluidity of the plasma membranes.

In the literature, there are only two reports that failed to show evidence for PMCA on MVM [13,14]. Accordingly, it is important to discuss the possible causes of the differences between the current report and the previous ones suggesting or showing that PMCA is only present in the BM. In one of them, Borke et al. [13], described by immunohistochemistry and Western blotting techniques with the monoclonal antibody 5F10, the presence of PMCA in both MVM and BM from hSCT, although the labeling of PMCA in MVM was really weak. It is clear that Borke et al. [13] could not rule out the presence of PMCA in MVM. Furthermore, none of them [13,14] used morphologic controls in their immunohistochemistry studies, e.g. staining of the nuclei. Therefore, in these studies it is not possible to affirm in a conclusive way, the presence of PMCA only in the fetal-facing side of hSCT.

Strid and Powell [14] showed, by means of immunocytochemistry techniques, a clear distribution of PMCA in the BM only. They also showed, by means of Western blot analyses, the presence of both PMCA1 and PMCA4 in the BM fractions only. However, they did not perform the same Western blot analyses for the MVM fractions. In contrast, in our Western blot studies using isoform-specific antibodies, PMCA4 but not PMCA1 was shown to be present in both MVM and BM (Fig. 4). Although it is difficult to try to explain the possible sources of differences between the study of Strid and Powell and our study, one can suggest that the source of the antibodies used in the current study is clearly different from that used by Strid and Powell, with the exception of the general PMCA, clone 5F10. Strid and Powell used a set of antibodies that differs in their specificity to the N-terminal region of each protein (first 80−90 amino acids). In our case, we used a polyclonal antibody against PMCA1, which was made using a synthetic peptide immunogen corresponding to the residues A(5) N N S V A Y S G V K N S I K E A N(22) of the rat PMCA1. This might account for some of the differences in distribution found for PMCA1 and PMCA4 in hSCT.

A careful revision of previous studies led us to find reports showing the presence of a high affinity Ca^{2+}-stimulated ATPase activity in MVM of human term placenta [35]. This ATPase activity was shown to be able to transport Ca^{2+} against its gradient in MVM vesicles [36], and to have biochemical characteristics resembling those of PMCA, e.g., high affinity for free Ca^{2+}, vanadate-sensitivity, calmodulin-stimulation, oxalate-insensitive, optimal pH around 7.2 [2]. Interestingly, these biochemical characteristics of the active transport of Ca^{2+} in MVM vesicles, are quite similar to those shown in Table 3 [36]. Furthermore, there is a report demonstrating the ultracytochemical localization of a Ca-ATPase activity in hSCT showing strong ATPase activity in MVM and a weak activity in BM [37], which is in agreement with the biochemical data (Table 3) and the Western blot analyses (Fig. 4) shown in the current study.

Our results lead us to propose a new model for the transport of calcium across the hSCT, considering the presence of an important fraction of PMCA in MVM. All the Ca^{2+} ions acquired by the fetus throughout gestation, must come necessarily from the maternal circulation and, since the Ca^{2+} concentration in the fetal blood is higher than that in the mother circulation [7], the ion must be actively transported across the placenta from the maternal to the fetal circulation. In fact, the syncytiotrophoblast actively transports close to 80% of the Ca^{2+} going from the maternal to fetal circulation, where it is needed for fetal skeleton growth, especially during the third trimester of pregnancy [8]. The directionality of the net transport of the ion across any of the individual plasma membranes will depend on the balance between the passive influx of Ca^{2+} and the rate at which the ion is pumped out across that membrane. Consequently, considering the fact that there is a higher activity of PMCA in MVM than in BM, the only way to have a net flux of the ion from the mother to the fetus is through a higher Ca^{2+} permeability in MVM than in BM. In fact, MVM possess more mechanisms of Ca^{2+} entry than those in BM, indicating a higher Ca^{2+} permeability in MVM than BM [8]. In this way, most of the Ca^{2+} entering passively to the syncytiotrophoblast across the MVM will be returned to the mother by the PMCA from these membranes. PMCA of BM, on the other hand, will be responsible for the quantity of Ca^{2+} extruded through BM, in order to get a net Ca^{2+} transport from the mother to the fetus. NCX does not seem to play an important role in the control of Ca^{2+} movement through hSCT [10].

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