

# Plasma membrane calcium ATPase isoform 3 expression in single cells isolated from rat liver

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**Abstract** The plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA) located in the hepatocyte is a controversial molecule in itself since it displays different features to those regarded as canonical for P-type  $\text{Ca}^{2+}$ -ATPases, and from which transcript expression as well as catalytic activity continues to be under active investigation. Our aim in this study was to explore at a first glance, *pmca* isoform distribution using isolated parenchymal and non-parenchymal cells from rat liver tissue. Expression of *pmca* transcripts was analyzed in fresh or cell-enriched culture preparations, confirming *pmca1* and *pmca4* as the housekeeping isoforms in all cell types studied (hepatocytes, Kupffer cells, and stellate cells). However, for the first time we show expression of *pmca3* transcripts edited at two different sites in both hepatocytes and non-parenchymal cells. Interestingly, employing non-parenchymal cells we demonstrate the specific expression of *pmca3e* transcripts previously considered nearly exclusive of excitable tissues. Real-time PCR quantification shows a significant decrease of *pmca3* transcripts in cultured Kupffer and hepatic stellate cells in

comparison with fresh cells. The presence of *pmca2* along with *pmca3* in all liver cell types studied suggests that high affinity isoforms are relevant to the adequate management of calcium in liver tissue, particularly when hepatic cells become activated by diverse stimuli.

**Keywords**  $\text{Ca}^{2+}$  homeostasis · Plasma membrane  $\text{Ca}^{2+}$ -ATPase · Hepatic parenchymal cells · Hepatic stellate cells · Kupffer cells · Single-cell RT-PCR

## Introduction

Metabolic homeostasis of the liver is maintained by the coordinated function performed by parenchymal (hepatocytes) and non-parenchymal cells. Non-parenchymal cells represent 20% of the hepatic mass and include Kupffer cells (KC), hepatic stellate cells (HSC), endothelial cells (EC), and lymphocytes. KC are resident macrophages that, in addition to performing phagocytosis of foreign molecules, produce cytokines as part of several immune responses [1]. HSC are devoted to the production of extracellular matrix and represent the most important site for storage of vitamin A in the organism. During liver regeneration, HSC and KC become activated, and begin to secrete growth modulators that are important in creating an optimal environment for recovery of the hepatic mass [2]. Activated KC showing evident morphologic changes are characterized by overproduction of inflammatory mediators and oxygen free radicals [3]. When HSC are activated in a chronic fashion, they play a relevant role in the process of liver fibrosis, exhibiting a myofibroblast-like phenotype in addition to overproduction of extracellular matrix proteins [4, 5].

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A diversity of functions depends on calcium as a basic second messenger in signal transduction pathways modulating liver homeostasis. For instance, KC phagocytosis is  $\text{Ca}^{2+}$ -dependent, and the presence of specific membrane proteins mobilizing calcium such as L-type channels and store-operated  $\text{Ca}^{2+}$  channels has been described [6, 7]. Likewise, after liver injury HSC activation related to intracellular  $\text{Ca}^{2+}$  regulation has also been observed [8]. Contrary to what is observed in hepatocytes, quiescent and activated HSC present voltage-operated  $\text{Ca}^{2+}$  channels [9, 10].

As we have previously reviewed, the plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA) seems to present a relevant function as a  $\text{Ca}^{2+}$  extrusion system in liver cells [11]. Four genes produce a large diversity of PMCA isoforms through a complex alternative splicing at two main sites: site A located near the phospholipid-sensitive domain, and site C located at the calmodulin (CaM)-binding domain of PMCA [12]. Although the presence in hepatic tissue of *pmca1* > *pmca4* > *pmca2* transcripts is well documented, much less knowledge is available considering *pmca* isoforms expression pattern in non-parenchymal cells [13, 14]. Expression of total PMCA in HSC caveolae and in EC fenestrae has been demonstrated by immunocytochemical methods [15, 16]. The presence of PMCA has been suggested in KC since a CaM-dependent  $\text{Ca}^{2+}$ -ATPase activity has been detected [17]. Nevertheless, specific PMCA isoforms expressed and their relevance in the phenomenon of activation in different liver cells remains unknown. Therefore, the aim of this study was to explore, as a first approach, *pmca* isoform transcript expression in isolated liver cells including hepatocytes, KC, and HSC immediately after isolation, as well as in primary cultures.

## Materials and methods

### Hepatocytes culture

Experimental animals were handled according to the Guide for the Care and Use of Laboratory Animals endorsed by the National Academy of Sciences (USA), in use in our Institute. Male Wistar rats weighing 250 g were anesthetized with an intramuscular injection of ketamine (40–80 mg/Kg) and xylazine (5–10 mg/Kg). Livers were perfused with a Krebs–Ringer solution (KR) containing 0.05% collagenase IV (Worthington, Lakewood, NJ, USA) [18]. Cell suspension was filtered and centrifuged at 500 rpm for 2 min; pellets were recovered and washed with KR to improve hepatocytes yielding.

For primary cultures, hepatocytes were resuspended in sterile PBS, diluted (v/v) with an isosmotic Percoll solution (45 mL Percoll/4.5 mL of 10× Hanks' balanced salt

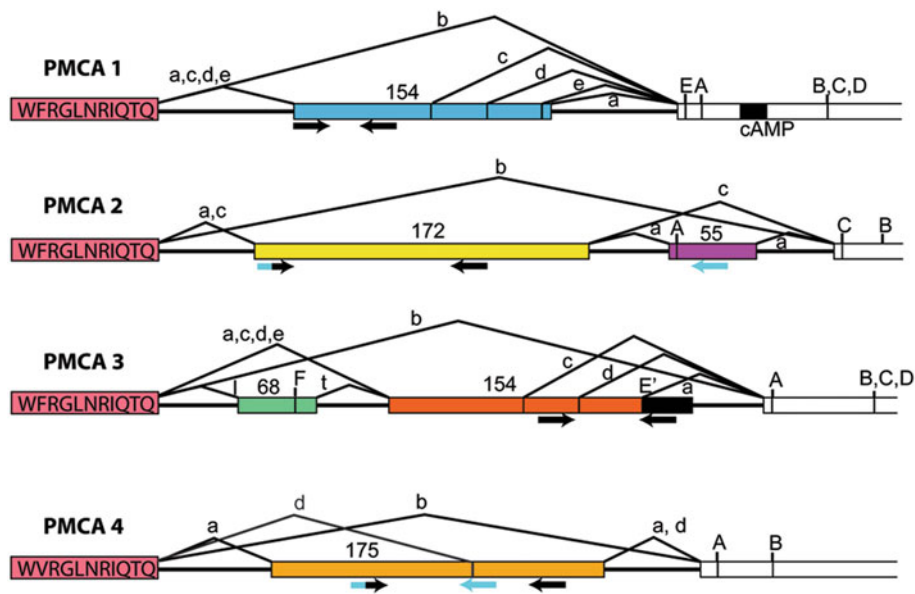
solution and 0.5 mL of 1 M Hepes) and centrifuged at 800 rpm for 5 min. Viabilities  $\geq 85\%$  were estimated by trypan blue exclusion. Hepatocytes were resuspended in Dulbecco's modified Eagle's medium (DMEM) plus 0.02% bovine serum albumin, 3 mM Hepes, 1 mM sodium pyruvate, 6 mM sodium bicarbonate, 1 mg/mL galactose, 0.2 mM proline, 4 mM L-glutamine, 10% fetal bovine serum (FBS), 5  $\mu\text{g}/\text{mL}$  insulin–transferrin–sodium-selenite (ITS), streptomycin (100  $\mu\text{g}/\text{mL}$ ), penicillin (100 U/mL), gentamicin (100 U/mL), and fungizone (0.25  $\mu\text{g}/\text{mL}$ ). Cells were plated in Petri dishes ( $2.5 \times 10^6$ ) and were allowed to attach during 3 h under a 5%  $\text{CO}_2/95\%$  air atmosphere at 37°C. The medium was then replaced for serum-free DMEM with no added growth factors until cell collection was carried out after 2 days. The estimated viabilities did not change after this period of time.

### Non-parenchymal cells culture

Male Wistar rats weighing 500 g were used for isolation of HSC and KC. Livers were perfused with KR and cells dissociated with 0.6–0.7% pronase and 0.15  $\mu\text{g}/\text{mL}$  collagenase type IV according to a modified method by Tsukamoto et al. [19]. Digested tissue was minced, incubated with 20  $\mu\text{g}/\text{mL}$  DNase I for 15 min at 37°C and separated in a Nycodenz gradient at 21,400 rpm for 45 min in a SW40 Ti rotor. HSC and KC were recovered from the 8.2 and 12% layers, respectively, and washed with PBS containing antibiotics and centrifuged at 2,500 rpm for 7 min. Cells were used immediately or placed in culture with a density of  $3.5 \times 10^6$  in DMEM supplemented with 10% FBS plus antibiotics under a 5%  $\text{CO}_2/95\%$  air atmosphere at 37°C. Culture medium was changed after 24 h and then every 2 days; the viabilities were as well ca. 85%.

### Polymerase-chain-reaction methods

Total RNA was isolated from different cell types using the Trizol reagent and treated with Turbo DNase (Ambion, Inc., Austin, TX, USA). Oligonucleotides for rat *pmca* isoforms spliced at site C (Fig. 1) and rat and/or mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were designed with the MacVector 6.5.3 software, or as previously reported [20–24] (Table 1). One-step RT-PCRs were started with 0.5  $\mu\text{g}$  of RNA in a final volume of 20  $\mu\text{L}$  containing 0.25  $\mu\text{M}$  of each primer (Invitrogen Carlsbad, CA, USA). Temperature steps were 50°C/30 min, 94°C/2 min for cDNA synthesis, followed by 40 cycles of the sequence 94°C/15 s, 55°C/30 s, 72°C/1 min, and a 10-min extension step. Markers for HSC and EC were amplified using 25 cycles [22, 23]. PCR products were separated in 4% ethidium bromide-stained agarose gels.



**Fig. 1** Primer design used for amplification of rat *pmca* transcripts spliced at site C. Conserved sequences coding for the constant region of CaM-binding domain are indicated (pink boxes) (24). Primers for *pmca1* recognize isoform “1c” (66 bp). Primers for *pmca3* align in the 154 bp exon (number 23) and in an extension of exon 23 (black box) producing variant “3e” (82 bp) [24, 31]. For *pmca2* and *pmca4*

two primer pairs were designed; forward primers were common to two alternative isoforms (blue/black arrows). Primers for *pmca2* recognize sites in the 172-bp exon of variant “2c” (132 bp) or variant “2a” (199 bp) including both exons. Primers for *pmca4* amplify two different fragments of variant “4a” (115 and 77 bp, respectively). (Color figure online)

**Table 1** Primers employed for RT-PCR analysis

Gene	Forward (5'–3')	Reverse (5'–3')	Size (bp)
<i>pmca1</i> [20]	GGCGACTTTGGCATCACACT	TTTCAACTTGGTGCAAATTCCA	120
<i>pmca2</i> [21]	AAGGAGACATATGGGGAC	TTCACCTTCATCTTCTGC	300
<i>pmca3</i> [21]	CACAGCCTTCAATGACTG	CCTTCCATGCATGAGTG	300
<i>pmca1c</i>	GGATGTAGTGAATGCTTTCCAG	ATGGAGGGTTGTCGCCTTAG	66
<i>pmca2c</i>	CAATACTTTCAAGAGCGGG	GGAGAGGAAAGAGCATTGGATAAC	132
<i>pmca2a</i>	CAATACTTTCAAGAGCGGG	TCTCTTGCTGAACTCTGTGTC	199
<i>pmca3e</i>	GTAACCAATCTTTCTACCCC	TTTACCACCTCGGATTGC	82
<i>pmca4a</i>	AGTCCTAAGGCGACAGAACTTGAG	TGTAACGGCAGAAGAGGTGGAG	115
<i>pmca4a'</i>	AGTCCTAAGGCGACAGAACTTGAG	ACGCAACTGCTTCTGAATAG	77
<i>PPAR<math>\gamma</math></i> [22]	ATTCTGGCCACCAACTTCGG	TGGAAGCCTGATGCTTTATCCCCA	332
$\alpha$ - <i>SMA</i> [22]	TGTGCTGGACTCTGGAGATG	GATCACCTGCCCATCAGG	292
<i>ELAM1</i> [23]	CAACGTGCACGTTTGACTGT	AGGTCAAGGCTTGAACACTG	508
<i>m/rGAPDH</i>	GGAGAAACCTGCCAAGTATGATGAC	TGGGAGTTGCTGTTGAAGTCG	126
<i>rGAPDH</i>	TCCCAGAGCTGAACGGGAAG	TTACTCCTTGAGGCCATGTAG	325

NCBI accession numbers: *pmca1*, NM\_053311; *pmca2*, NM\_012508; *pmca3*, NM\_133288; *pmca4*, NM\_001005871

### Single-cell RT-PCR

Single hepatocytes as well as KC and HSC were harvested under a differential interference contrast (DIC) infrared microscope (Nikon FN1) from <24-h cultures employing a whole-cell configuration and recovered applying negative pressure into the glass pipette. Each single cell was collected in sterile tubes containing RNase inhibitors, frozen

in liquid nitrogen and kept at  $-70^{\circ}\text{C}$  until use. Isolated hepatocytes were treated with DNase I and used immediately for RT-PCR with the AccessQuick<sup>TM</sup> system (Promega Co., Madison, WI, USA) using 50 cycles for amplification as reported by us [25]. PCR products were separated in 3% agarose-TBE gels, soaked in SYBR Safe stain (Invitrogen, Carlsbad, CA, USA) for 1 h, and analyzed in a Typhoon 9400 scanner (GE Healthcare).

## Real-time RT-PCR

Specific primers for *pmca3* (NM\_133288), forward 5'-GCAGATGTTGTGGGTGAACT-3', reverse 5'-CGTGAGATGAGAGGCTTGTC-3', and the Taqman probe CTTCCGCA GCAGCAGTGA CTCTGTTG were designed (Primer Design Ltd, Southampton, UK). One-step quantitative-PCR assays using total RNA (0.5 µg) from fresh and cultured hepatocytes, KC and HSC were performed according to the following program: 50°C/15 min, 95°C/2 min and 40 cycles at 95°C/15 s, 60°C/40 s in an ABI Prism 7000 detection system from Applied Biosystems (Foster City, CA, USA).

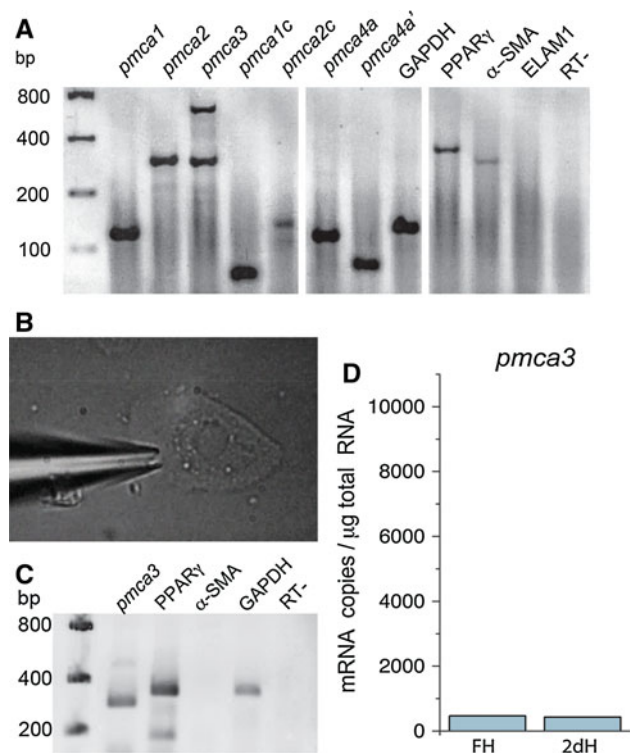
## Results

### *pmca* transcripts in hepatocytes

*pmca1* and *pmca4* isoforms are abundantly expressed in fresh hepatocytes (Fig. 2a) and those maintained for 2 days in culture (data not shown). Since the use of end-point PCR techniques did not allow us to discriminate differences in the expression pattern between fresh and 2-day hepatocytes, we only show results using fresh cells. In general terms, *pmca2* transcripts edited upstream of site A (UA) and exhibit a higher level of expression than those spliced at site C. We consistently found the expected *pmca3* isoform (UA, 300 bp) and a second band (~650 bp) identified as a member of the toll-like receptor family (TLR3), which has been found in hepatocytes and KC [26, 27]. Products obtained with primers designed for *pmca2a* and *pmca3e* (site C) showed low concentration bands that did not correspond to the expected size, therefore they were not included in the corresponding figure (Fig. 2a).

In addition, we show in hepatocytes the presence of the *peroxisome proliferator-activated receptor*  $\gamma$  (*PPAR* $\gamma$ ) and  $\alpha$ -*smooth muscle actin* ( $\alpha$ -*SMA*) transcripts described as markers and whose expression specifically diminishes or becomes induced in activated HSC [22]. In contrast, the marker for EC, *E-Selectin* (*ELAM-1*) [23] was absent (Fig. 2a). Since some contamination from non-parenchymal cells during hepatocyte isolation is feasible, we decided to collect single hepatocytes (Fig. 2b) and confirmed the presence of *pmca3* (UA) and *PPAR* $\gamma$  transcripts using a system detecting targets at the zeptomolar level (Fig. 2c). In our hands, employing isolated hepatocytes it was not possible to amplify transcripts <200 bp including rat/mouse *GAPDH* (126 bp); therefore, rat *GAPDH* primers yielding 325-bp amplicons were used (Table 1).

Using real-time PCR as the most sensitive technique available, the *pmca3* isoform (site C) was quantified in

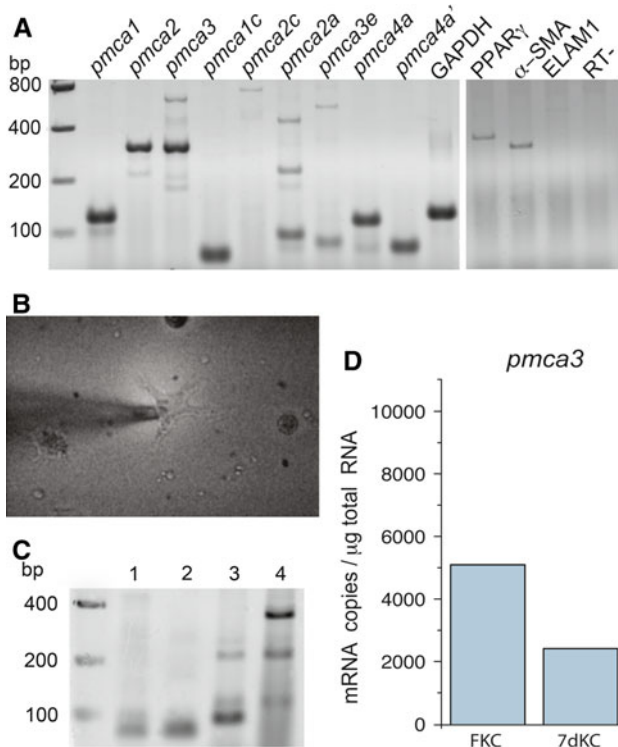


**Fig. 2** Analysis of *pmca* transcripts expressed in hepatocytes. **a** Representative experiment showing *pmca* transcripts of edited upstream of site A (*pmca1-3*), and site C from fresh hepatocytes-enriched preparations (FH). **b** Single hepatocyte harvested under a DIC infrared microscope in a whole-cell configuration. **c** Sybr Safe-stained gel showing transcripts from single independent hepatocytes, confirming expression of *pmca3* and *PPAR* $\gamma$ . **d** Representative experiment (from three experiments) using triplicates showing real-time PCR quantification of *pmca3* transcripts (site C) in FH and hepatocytes placed 2 days in culture (2dH). Mean values  $\pm$  SE in FH ( $470.37 \pm 0.85$ ) and in 2dH ( $429 \pm 0.83$ ). The y axis scale is the same as in Figs. 3d and 4d

fresh and cultured hepatocytes showing no apparent differences (Fig. 2d).

### *pmca* transcripts in Kupffer cells (KC)

In KC, the most abundant *pmca* transcripts show to be the housekeeping isoforms *pmca1* and *pmca4* spliced in both explored sites, followed by the *pmca2* and *pmca3* isoforms (UA, Fig. 3a). *TLR3* transcripts were also detected in amplifications with primers for *pmca3* (UA). A slight ~199-bp band corresponding to *pmca2a* transcripts was visualized in addition to several unspecific transcripts. Interestingly, *pmca3e* was detected under all conditions and its identity was determined by automated sequencing. Transcripts corresponding to *PPAR* $\gamma$ ,  $\alpha$ -*SMA* and to a lesser extent *ELAM-1* were evident in KC fractions. The presence of *pmca3e* transcripts was confirmed in single KC (Fig. 3c).

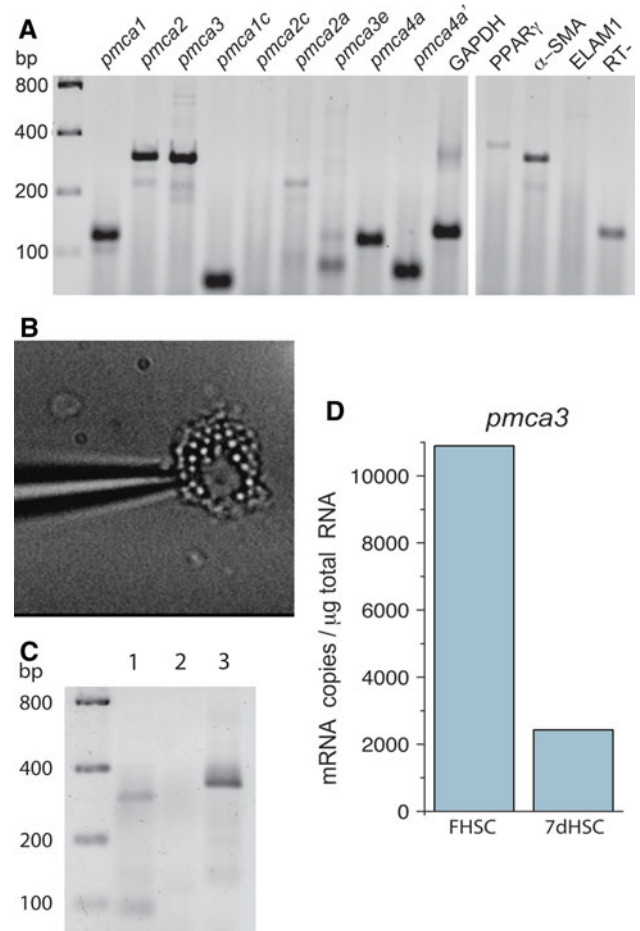


**Fig. 3** Analysis of *pmca* transcripts expressed in Kupffer cells. **a** Representative experiment showing *pmca* transcripts edited upstream of site A (*pmca1-3*), site C, together with activation markers from fresh KC-enriched (FKC) preparations. **b** Single KC harvested under a DIC infrared microscope in a whole-cell configuration. **c** Sybr Safe-stained gel showing *pmca3e* transcripts (82 bp) expressed in a single fresh KC (lane 1); in 10 isolated KC (lane 2); in fresh KC-enriched preparations (lane 3) and KC after 7 days in culture (7 days KC, lane 4). **d** Representative experiment (from three experiments) using triplicates showing real-time PCR quantification of *pmca3* transcripts (site C). Mean values  $\pm$  SE in FK (5,116.78  $\pm$  1.19) and in 7 days KC (2,439.78  $\pm$  0.80). The y axis scale is the same as in Figs. 2d and 4d

Since we observed apparent changes in expression of *pmca3e* in KC cultured for 7 days (Fig. 3c), we further explored *pmca3* using real-time PCR techniques. In experiments with total RNA obtained from KC-enriched preparations, after 7 days in culture we observed a lower level of *pmca3* expression (Fig. 3d).

*pmca* transcripts in hepatic stellate cells (HSC)

In HSC, a similar expression pattern to that of hepatocytes and KC for *pmca1* and *pmca4* isoforms can be observed (Fig. 4). In addition, the expected amplicon for *pmca3* (UA) was observed with a higher level of expression, in contrast to that spliced at site C. In a similar fashion to that observed in KC, *pmca2c* (132 bp) is absent, while *pmca2a* (~199 bp) is slightly expressed (Table 2). *PPAR $\gamma$*  and  $\alpha$ -*SMA* transcripts were always observed in fresh HSC, while *ELAM-1* transcripts were absent (Fig. 4). At the



**Fig. 4** Analysis of *pmca* transcripts expressed in hepatic stellate cells (HSC). **a** Representative experiment showing *pmca* transcripts edited upstream of site A (*pmca1-3*), site C together with activation markers from fresh HSC. **b** Single-HSC harvested under a DIC infrared microscope in a whole-cell configuration, showing the typical vitamin A droplets associated to this cell type. **c** Sybr Safe-stained gel showing transcripts using total RNA (200 ng) from fresh HSC; lane 1, *pmca3* edited upstream site A (300 bp); lane 2, *pmca3e* (site C), non-detected; lane 3, *GAPDH* (325 bp). **d** Representative experiment (from three experiments) using triplicates showing real-time PCR quantification of *pmca3* transcripts (site C). Mean values  $\pm$  SE in FK (5,116.78  $\pm$  1.19) and in 7 days KC (2,439.78  $\pm$  0.80). The y axis scale is the same as in Figs. 2d and 3d

**Table 2** Summary of *pmca* transcripts expressed in different cell types contained in the liver

Cell type	Site UA			Site C					
	1	2	3	1c	2c	2a	3e	4a	4d'
Hepatocytes	++	++	++	++	+	-	-	++	++
Kupffer cells	++	++	++	+	-	+	+	++	++
Stellate cells	++	++	++	++	-	+	+	++	++

++ high level of expression, + moderate expression, + low level of expression, - absent or showing a different size

single-cell level, *pmca3e* transcripts were out of the detection limits of our amplification system (AccessQuick™), and therefore only found in experiments using 100–200 ng of RNA isolated from several HSC (Fig. 4c). However, the detection of *pmca3* (site C) transcripts was accomplished in single-HSC using real-time PCR with an expression of  $\leq 10$  mRNA copies/cell. Further experiments using real-time PCR and total RNA isolated from HSC, showed a diminished level of expression of *pmca3* in cells maintained in culture for 7 days (Fig. 4d).

## Discussion

To date, relevant proteins involved in  $\text{Ca}^{2+}$  homeostasis at the intracellular and extracellular levels together with the expression of a G-protein-coupled receptor that senses extracellular  $\text{Ca}^{2+}$  levels in the hepatocyte are well known [28, 29]. Nevertheless, the participation of the several  $\text{Ca}^{2+}$  extrusion systems described in liver cells such as the PMCA and the NCX are less understood [11]. For the first time, our study explores *pmca* isoform expression in liver using freshly isolated and cultured hepatocytes, KC- and HSC-enriched preparations. Analysis of *pmca* transcript expression in human and rat liver show the presence of *pmca1*, followed by *pmca4*, and *pmca2* [13, 14]. However, the presence of *pmca3* transcripts has not been demonstrated in liver yet, probably due to the use of degenerate primers and the complex splicing pattern displayed by *pmca* encoding genes [30]. The important expression of *pmca3* transcripts (UA) and the moderate expression of *pmca3* (site C) in all liver cell types studied was confirmed at the single-cell level using either a sensitive end-point PCR kit (AccessQuick™) or through real-time PCR.

Interestingly, *pmca3e* transcripts were only observed in non-parenchymal cells (Figs. 3, 4; Table 2). The 3' end of these transcripts aligns to a sequence located in an 88-bp extension of the 154-bp exon (exon 23) included in exon 24 (Fig. 1), which is expressed in rat skeletal muscle as well as in brain mRNAs [31]. In the human, this variant is present in fetal skeletal muscle [13] and the complete sequence of *pmca3* is also reported in brain [32]. Exon 24 can be translated according to two reading frames depending upon the upstream splice site, followed by an alternative polyA signal. The presence of acidic (A) or basic (B) CaM-binding domains in the PMCA expressed possesses important physiological implications, such as the type B CaM-binding site which displays a significantly higher affinity for CaM (up to 10-fold) and a higher autoinhibitory capacity (15-fold) than the type A CaM-binding site [33]. Since the CaM-binding site encoded by exon 24 corresponds to a B form, the physiological

relevance for the presence of these *pmca3* transcripts in non-parenchymal cells is open to investigation.

It is noticeable that real-time PCR using primers detecting *pmca3* (site C) were able to detect transcripts in different RNA preparations from hepatocytes that in comparison to non-parenchymal cells were less abundant (Fig. 2d). Moreover, this isoform seems to be non-relevant when studied in fresh or 2-day cultured hepatocytes. This might reflect the fact that under these conditions cells never have been under the effect of added growth factors that trigger replication in vitro and dedifferentiation normally observed in long-term cultured hepatocytes.

Data from real-time PCR show a 50% decrease in the level of expression observed for *pmca3* in KC cultured for 7 days in comparison to fresh KC (Fig. 3d). Although primary cultures of KC are not considered to be involved in a possible activation process by itself, based on published evidence we believe this might be the case. Since it has been recognized that non-parenchymal cells might become activated during the cell's isolation procedure [34], we believe this phenomenon is occurring with our KC placed in culture. Taking into consideration that KC become activated after  $\text{CCl}_4$ -treatment or as a result of exposition to bacterial extracts showing an important induction of osteopontin mRNA expression [35, 36], we searched for osteopontin transcripts in our KC preparations. We found that osteopontin is expressed in both fresh and cultured KC suggesting that the process of activation is taking place after KC isolation (data not shown). Since our group has recently correlated the overexpression of osteopontin with the process of atherosclerosis through the induction of a state of oxidative stress (Jiménez-Corona et al., submitted), this phenomenon might be related to the activation of our KC and therefore it will be further studied.

On the other hand, one interesting although controversial hypothesis suggests a neuroendocrine origin for HSC based on the fact these cells share the expression of several markers observed in nervous system cells [37–39]. The presence of *pmca3e* transcripts in HSC seems to reinforce this notion, since the possibility that nerve endings might be contaminating HSC preparations [40] was discarded with the use of single-HSC experiments (Fig. 4b) where *pmca3* (site C) was quantitatively measured.

Since PMCA3 and PMCA2 are considered isoforms that show the highest affinities for  $\text{Ca}^{2+}$  and CaM reported among the four PMCA isoforms, it will be interesting to elucidate in non-parenchymal hepatic cells the function associated to these “fast” isoforms (mainly present in excitable tissues). For several PMCA2 isoforms spliced at site A and for PMCA3a in the presence of CaM, apparent  $K_m \text{Ca}^{2+}$  constants range between 0.3–0.5  $\mu\text{M}$  and  $K_m \text{CaM}$  up to 10 nM [41, 42]. The highest affinities comprise those from PMCA2 variants spliced at site C, 2a

( $K_m$   $Ca^{2+}$  0.09  $\mu$ M/ $K_m$  CaM 8.4 nM), and 2b ( $K_m$   $Ca^{2+}$  0.06  $\mu$ M/ $K_m$  CaM 2.1 nM) [43]. In contrast,  $Ca^{2+}$  affinity for PMCA4b is ca. 0.5  $\mu$ M, and CaM affinity ca. 50–100 nM [41]. These contrasting kinetic properties are closely related with the ability of individual PMCA isoforms to restore the intracellular calcium concentration after an inward movement of calcium has occurred [44]. Since non-parenchymal cells play a relevant role in supporting hepatocyte survival after diverse stimuli, efficient PMCA isoforms must be readily available to maintain  $Ca^{2+}$  homeostasis in the whole organ. In this respect, it is important to note that activated HSC showing contractile activity [45, 46] must also require the presence of PMCA to modulate the intracellular levels of calcium and therefore their contractile function.

In order to understand the genotype changes that seem to affect different liver cells or the way in which the post-activation process could be interpreted, a temporal course of changes after seeding cells in culture must be analyzed. Also, the complete analysis of expression changes occurring with other *pmca* variants that could counteract the specific decrease of *pmca3* transcripts detected in cultured KC will be carried out. The present study in the near future will help us to solve many of the questions still unanswered related to the function of the dynamic changes in the levels and isoform type of the plasma membrane calcium pump in non-excitable cells.

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