

Is there a specific role for the plasma membrane Ca^{2+} -ATPase in the hepatocyte?

Blanca Delgado-Coello, Raquel Trejo and Jaime Mas-Oliva

Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México, D.F. México

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Abstract

The plasma membrane Ca^{2+} -ATPase (PMCA) is responsible for the fine, long-term regulation of the cytoplasmic calcium concentration by extrusion of this cation from the cell. Although the general kinetic mechanisms for the action of both, well coordinated hydrolytic activity and calcium transport are reasonably understood in the majority of cell types, due to the complex physiologic and biochemical characteristics shown by the hepatocyte, the study of this enzyme in this cell type has become a real challenge. Here, we review the various molecular aspects known to date to be associated with liver PMCA activity, and outline the strategies to follow for establishing the role of this enzyme in the overall physiology of the hepatocyte. In this way, we first concentrate on the basic biochemical aspects of liver cell PMCA, and place an important emphasis on expression of its molecular forms to finally focus on the critical hormonal regulation of the enzyme. Although these complex aspects have been studied mainly under normal conditions, the significance of PMCA in the calcium homeostasis of an abnormal liver cell is also reviewed. (*Mol Cell Biochem* **285**: 1–15, 2006)

Key words: PMCA, Ca^{2+} homeostasis, hepatocyte, hormone regulation

Introduction

The plasma membrane Ca^{2+} -ATPase (PMCA) pumps cytosolic calcium to the extracellular space and presents a major role in long-term control of intracellular free Ca^{2+} levels in eukaryotic cells [reviewed in 1–3]. The majority of PMCA properties have been determined using human erythrocyte ghosts and later confirmed utilizing the purified enzyme [4–8]. ^{45}Ca -uptake and $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase kinetic constants as well as the mechanisms by which erythrocyte PMCA activity is controlled, became the reference to compare with the Ca^{2+} -ATPase activity associated with both the microsomal membrane fraction, and ATPases from other cell types. Although some tissues show high Ca^{2+} -ATPase activity similar to the erythrocyte Ca^{2+} pump, other tissues such as liver, evidenced a more complex situation. Liver PMCA kinetics present major differences with respect to the erythro-

cyte enzyme, the most relevant being regulated by hormones through membrane-associated proteins.

The main goal of this review was to analyze the knowledge generated throughout the years concerning the main properties and regulatory mechanisms that control the plasma membrane Ca^{2+} -ATPase present in the hepatocyte under normal and pathological conditions. Divided in three main sections, i.e., Biochemistry, molecular forms expression, and hormonal regulation; this review attempts to set forth a critical evaluation of the observations made by many scientists over the years to define whether there is a specific role for plasma membrane Ca^{2+} -ATPase in the hepatocyte.

Biochemistry of liver PMCA

Isolation and purification of a plasma membrane fraction free of contaminating Ca^{2+} -ATPases derived from mitochondria

and endoplasmic reticulum vesicles was the first problem to solve for characterization of liver PMCA. Several strategies have been used to homogenate and fractionate the tissue using hypotonic [9–27], isotonic [13, 28–39], or hypertonic [40–42] solutions, supplemented with calcium [11, 13, 24, 29, 39], with EGTA or in the absence of added calcium [31–33, 35–37]. Surprisingly, addition of protease inhibitors during liver fractionation was not reported in the majority of these studies, with the exception of the work reported by Kessler *et al.* [38]. Plasma membrane vesicles have been purified mainly from nuclear or microsomal fractions by centrifugation using sucrose or Percoll density gradients. However, contamination with ER membranes determined by the 5'-nucleotidase/glucose-6-phosphatase activity ratio was variable [24, 35, 41, 42].

The second problem to solve was separation of the sinusoidal, and canalicular regions of plasma membrane [13, 14, 43, 44] to define whether PMCA is restricted to a specific functional domain of the hepatocyte [24, 26, 38].

Ca²⁺-ATPase activity in liver plasma membrane vesicles

A high-affinity Ca²⁺-ATPase has been consistently observed by many authors in liver plasma membrane vesicles; however, the reported V_{max} values fluctuate from 33 [17] to 1,300 nmoles Pi mg protein⁻¹ min⁻¹ [32], and the apparent K_a for Ca²⁺ from 25 nM [24] to 1.6 μM [32]. Ca²⁺-ATPase activity shows a sigmoid curve when assayed in the presence of different substrate concentrations. An apparent K_m of 200 μM was obtained considering free ATP as substrate, but a K_m of 4 μM was reported when Mg-ATP was used instead [37]. Endogenous nanomolar levels [32, 37] or exogenous micromolar concentrations of Mg²⁺ stimulates Ca²⁺-ATPase activity, nevertheless, millimolar Mg²⁺ concentrations inhibit this activity [41, 42].

The kinetic constants reported for ⁴⁵Ca-uptake also have shown important variations. The apparent affinity for Ca²⁺ fluctuates from 17 nM [17] to 1–6 μM [32, 37], and V_{max} values range from 35 pmoles [17] to ca. 4 nanomoles Ca²⁺ mg protein⁻¹ min⁻¹ [32, 37]. Calcium transport shows two apparent affinities for ATP, where K_m for the high-affinity site corresponds to 81 μM and for the low-affinity site, 10 mM [17]. A single K_m value of 150 μM for both free ATP and Mg-ATP complex has been also reported [37]. Addition of millimolar concentrations of Mg²⁺ has appeared necessary for ⁴⁵Ca-uptake to take place [17, 24, 26, 30, 31, 36–39].

The great variability of kinetic data between the papers mentioned previously could be explained considering the different procedures employed to isolate liver plasma membrane vesicles and the methods used to measure both Ca²⁺-ATPase activity and ⁴⁵Ca-uptake. Moreover, variables such

as age, weight, and sex of experimental animals as well as feeding conditions also could have influenced the results. Whether hepatocyte plasma membrane domains show significant differences in PMCA activity or not remains controversial, because studies using plasma membrane sub-fractions claiming to be enriched in blood sinusoidal, lateral or canalicular membrane regions show discrepancies attributable to the highly variable methodology conditions employed [24, 26, 38]. It should be pointed out that the canalicular region located in close proximity to the endoplasmic reticulum containing abundant inositol 1,4,5-triphosphate receptors (IP₃R) [43] accounts for ca. 10% of the total hepatocyte plasma membrane. The canalicular domain is free of Na⁺-K⁺-ATPase and glucagon-stimulated adenylate cyclase activity and shows to be enriched in leucyl-naphthylamidase, γ-glutamyl-transpeptidase, 5'-nucleotidase, alkaline phosphatase, and Mg²⁺-ATPase activities [13, 14, 44, 45]. Sialic acid, sphingomyelin, and cholesterol content in the bile canalicular domain doubles the levels found in blood sinusoidal and lateral regions [44]. Because cholesterol has been shown to modify the activity of cardiac muscle PMCA [46–48] drastically, it remains to be defined whether this lipid affects the PMCA contained in these regions. On the other hand, blood sinusoidal and lateral membrane domains represent 90% of total plasma membrane area, and contain high Na⁺-K⁺-ATPase and glucagon-stimulated adenylate cyclase activities [44, 45].

Ca²⁺-ATPase activity in erythrocyte ghosts demonstrates a V_{max} of 33 nmoles Pi mg protein⁻¹ min⁻¹, an apparent K_{Ca} of 2 μM, and K_m values for ATP of 3.5 and 120 μM for high and low-affinity sites, respectively. Inside-out red blood cell membrane vesicles transport approximately 13 nmoles Ca²⁺ mg protein⁻¹ min⁻¹, and requires the addition of millimolar Mg²⁺ [49]. Based on this information, it can be concluded that erythrocyte and liver PMCA show similar properties when assayed in their original membrane microenvironment. An increased sensitivity to Mg²⁺ shown by liver Ca²⁺-ATPase is the only important difference found to date with respect to the erythrocyte enzyme.

Partially purified liver PMCA

To clearly define its properties, Ca²⁺-ATPase has been partially purified from liver plasma membrane using gel filtration chromatography [15, 16, 19–21, 23, 25, 42], and incorporated into liposomes [20, 25]. Several experiments using this type of preparations showed a K_a in the nanomolar range [15, 19, 25] and a V_{max} that fluctuated within a small range from 0.120 [23] to 0.750 [16] μmoles Pi mg prot⁻¹ min⁻¹. Micromolar levels of Mg²⁺ stimulate ATPase activity [15, 20, 42] in contrast to millimolar concentrations, which inhibit the enzyme [15]. These results confirmed previous observations

using plasma membrane vesicles with the enzyme located in its natural microenvironment [32, 37, 40, 42].

The molecular weight of partially purified Ca^{2+} -ATPase shows important variations depending on the method employed. For instance, a molecular weight of 200 kD has been defined by filtration on Sephadex G-200 [19, 41]. Moreover, when Stokes radius and sedimentation coefficients are considered, the molecular weight of the protein corresponds to 140 kD, and by SDS-PAGE, 120 kD [42] and 70 kD [19]. However, the molecular weight of the Ca^{2+} -dependent vanadate-inhibited phosphorylated protein corresponded to 118 kD [20]. It is important to mention that none of these results were obtained using protease inhibitors during enzyme purification.

On the other hand, presence of an ecto-ATPase stimulated by Ca^{2+} and Mg^{2+} in a crude plasma membrane fraction of the liver [19–21] has originated an intense debate [25, 37, 50]. These ecto-ATPases are neither CaM-activated nor vanadate-inhibited [21, 50]. The plasma membrane ecto-ATPase has been purified [51], and through the production of antibodies, localized for instance in cardiac muscle [52]. These antibodies do not cross-react either with classical PMCA or with sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA). This ecto-ATPase has been also localized in tissues such as kidney, and skeletal muscle, as well as brain. It has been reported to present homology with two membrane glycoproteins: a hepatic cell-adhesion molecule called cell-CAM 105, and with protein pp120/HA4, recognized by the insulin receptor tyrosine kinase [53]. Moreover, presence of extracellular ATP as a substrate for this ecto-ATPase has suggested its involvement with several metabolic pathways such as gluconeogenesis and ureagenesis [54]. Because this ecto-ATPase releases ADP from ATP in the extracellular space, and because ADP is a ligand for the P2Y purinergic family of receptors that interact with G proteins [55], association of these receptors with modulation of PMCA in the hepatocyte remains to be elucidated. In human hepatocytes, it has been reported that P2Y receptors control both glycogen metabolism and several proliferation-associated responses [56].

Affinity purified liver PMCA

Moore and Kraus-Friedmann [30] partially purified a liver Ca^{2+} -ATPase by CaM-affinity chromatography from CaM-depleted, Triton X-100 solubilized microsomal membrane fractions. Ca^{2+} -ATPase activity shown by the purified protein was stimulated by micromolar levels of Ca^{2+} , presented dependency on Mg^{2+} and was stimulated by addition of exogenous CaM; nevertheless, TFP partially inhibited this calmodulin effect exerted upon Ca^{2+} -ATPase activity [30]. Unfortunately, no further purification nor characterization of

the isolated enzyme was carried out in this study to determine whether this partially purified Ca^{2+} -ATPase was conclusively the liver PMCA.

Using monoclonal antibody 5F10 directed toward a cytoplasmic sequence present in all PMCA isoforms [57], Kessler *et al.* [38] purified the liver PMCA to homogeneity, reported a molecular weight of 140 kD and for the first time studied its immunolocalization. They showed that blood sinusoidal domain is enriched with PMCA, while lateral and canalicular regions present a lower PMCA density.

Immuno-electron microscopy studies have shown that stellate and sinusoidal endothelial cells express PMCA [58, 59]. In this case, PMCA was localized in caveolae of stellate cells and in fenestrae of endothelial cells.

CaM-overlay experiments of purified liver PMCA showed that liver Ca^{2+} -ATPase presents a higher affinity for CaM binding than the erythrocyte enzyme. Although a masked CaM binding domain in liver PMCA has been postulated to be accessible under denaturing experimental conditions [38], the high affinity for Ca^{2+} shown by liver PMCA could have been caused by endogenous CaM already bound to liver PMCA. This possibility is supported by the observation that in presence of CaM, the affinity for Ca^{2+} shown by the erythrocyte PMCA changes from the μM to the nM range [3]. PMCA maximal activity can also be stimulated by acidic phospholipids [60, 61], phosphorylation [62, 63], self-association [64], and mild proteolysis [65]. Phospholipids interact with a sequence of PMCA toward the NH_2 end [66]. The phosphorylation site is localized near the carboxy terminal sequence of the enzyme and constitutes part of the PMCA regulatory region. It comprehends ca. 100 residues for the “a” isoforms and 150 aminoacids for the “b” isoforms [reviewed in 67].

On the other hand, CaM-binding domain in hPMCA1, the predominant isoform expressed by liver under normal and pathological conditions [68–70], presents the following sequence: LRRGQILWFRGLNRIQTQIRVVNAFRSS [71]. This sequence allows liver PMCA to bind calmodulin with high affinity [72, 73]. It is possible that CaM bound to PMCA1b could render unavailable the phosphorylation sequence to PKA or PKC [74], dimerization [75], as well as proteolytic activation of PMCA [76, 77].

Phosphatidylserine, a phospholipid able to activate erythrocyte PMCA and capable of preventing further stimulation by CaM [78], is distributed only on the inner side of the plasma membrane of both blood sinusoidal and bile canalicular domains of hepatocytes [45], therefore, liver PMCA localized in these domains could be considered constantly phosphatidylserine-activated. However, canalicular membranes that contain high cholesterol levels might counteract the effects of phosphatidylserine, because high cholesterol/phospholipid ratios have been shown to inhibit PMCA activity [47, 79, 80]. On the other hand, since liver expresses

the splice variant *pmca1* [69, 81], splicing at this site affects both the sequence contiguous to the phospholipid-sensitive region and also the sequence involved in PMCA inhibition [67].

Inhibition of liver PMCA

Taking advantage of the inhibitory effect of vanadate on the P-type class of ion motive ATPases [82, 83], this compound has been used to characterize liver PMCA. In presence of millimolar Mg^{2+} and K^+ , both Ca^{2+} -ATPase and ^{45}Ca -uptake of liver plasma membranes are shown to be very sensitive to vanadate. Values of vanadate $K_i \leq 1 \mu M$ for ^{45}Ca -uptake have been reported by several authors [36, 37], whereas $1 \mu M$ vanadate induced an inhibition of 95% of Ca^{2+} -ATPase activity [38]. When ^{45}Ca -uptake was assayed in absence of K^+ but using millimolar Mg^{2+} , vanadate K_i increased up to $20 \mu M$ [17]. A diminished inhibitory effect of vanadate was observed when Ca^{2+} -ATPase was assayed using micromolar levels of Mg^{2+} [24]. In absence of both Mg^{2+} and K^+ , vanadate presents no effects even at 2 mM concentration [41, 42]. Erythrocyte Ca^{2+} -ATPase activity and ^{45}Ca -uptake were maximally inhibited by vanadate when incubated with millimolar Mg^{2+} and K^+ showing a $K_i = 5 \mu M$ [84, 85]. Therefore, erythrocyte and liver PMCA again showed no differences toward vanadate sensitivity.

Lanthanum (La^{3+}) specifically inhibits turnover of the phosphorylated catalytic intermediate of PMCA, producing a decrease in both Ca^{2+} -ATPase and ^{45}Ca -uptake. Because it has no effect on other P-type ion-transport ATPase, it allows identification of PMCA even in crude membrane fractions [82, 86–89]. Lead (Pb^{2+}) has been also shown to inhibit erythrocyte PMCA interfering with the formation of phosphorylated intermediates [90, 91]. On the other hand, Ca^{2+} -dependent hydroxylamine and La^{3+} -sensitive protein phosphorylation have been assayed by several authors using liver plasma membrane vesicles [17, 24, 26, 38]. Molecular weight of the phosphorylated PMCA intermediate varies from 105 [17] and 200 kD [24], with intermediate values of 135 [36] to 140 kD [38] for the phosphoenzyme from basolateral plasma membrane vesicles. Molecular weight of the phosphorylated intermediate determined in erythrocyte plasma membrane vesicles using La^{3+} corresponds to 140 kD [88]. Again, this value is similar to the apparent molecular weight of the liver phosphoenzyme detected in membrane vesicles from the basolateral domain obtained in presence of protease inhibitors [38]. Because endogenous proteolytic activity was not blocked during isolation and purification of plasma membrane proteins in many of the studies performed to date, it appears very likely that phosphoproteins showing low molecular weight might represent proteolytic products of phosphorylated PMCA.

Effect of exogenous CaM, anti-CaM drugs and novel molecules on PMCA

Because ^{45}Ca -uptake and Ca^{2+} -ATPase activity present in erythrocyte ghosts are stimulated 3–5 fold by exogenous CaM and this stimulation is blocked by several anti-CaM drugs [1, 2], these parameters also have been assayed to characterize liver Ca^{2+} -ATPase. It has been commonly observed that liver Ca^{2+} -ATPase and ^{45}Ca -uptake are not stimulated by addition of CaM in both plasma membrane vesicles [24, 26, 32, 38, 41, 42], nor are they inhibited by anti-CaM drugs such as R24571 [39], trifluoroperazine (TFP) and W7 [26, 39, 41]. These properties were also observed with the partially purified enzyme using gel filtration chromatography [15–19].

Several factors might explain lack of stimulation by exogenous CaM; first, PMCA could have been activated by endogenous phosphatidylserine [13, 14], an acidic phospholipid known to activate erythrocyte PMCA [92]. Second, because no protease inhibitors were used during membrane and protein purification, elimination of the autoinhibitory sequence by endogenous proteases might have activated the PMCA [76, 77, 93]. Although TFP could have blocked activation of PMCA by proteolysis, by phosphatidylserine and by CaM [94], the concentration required to achieve this was higher than the level normally used [26]. The compound R24571 is able to remove the stimulation induced by phosphatidylserine if CaM is absent [94]. Mellitin, the anti-CaM peptide, inhibited Ca^{2+} -ATPase activity up to 80%, suggesting a direct interaction between mellitin and PMCA [24].

V_{max} and Ca^{2+} affinity of PMCA are increased by PKA and PKC phosphorylation [reviewed in 3, 95]. All PMCA isoforms show the sequence for PKC phosphorylation that occurs at a single threonine in the CaM binding domain [96]. Phosphorylation of serine 1178 by PKA has also been shown [97]; nonetheless, this site is only present in the PMCA1 isoform [3]. In different cell types expressing PMCA1 isoform, Ca^{2+} -ATPase activity was stimulated by PKA and PKC [98–100]; however, activation of Ca^{2+} -ATPase activity by PKC or PKA has not been observed in liver PMCA. The membrane microenvironment also has been postulated as a factor involved in the refractory behavior of hepatocytes toward PKC activation [95].

Regucalcin has been suggested as a stimulating protein of liver Ca^{2+} -ATPase [101]. Experimental evidence indicates that this protein interacts with membrane lipids and with sulfhydryl groups contained in Ca^{2+} -ATPase. It potentiates the effects of DTT and protects liver PMCA from N-ethylmaleimide (NEM) inhibition [102–104]. Regucalcin action is also dependent on intact membrane structure, because digitonin-treated membranes do not recover normal activity by incubation with this protein [102–104]. It has also been postulated as a regulatory molecule in the Ca^{2+} signaling pathway in regenerating liver [104, 105] as well

as in hepatoma cells [106, 107]. In regenerating liver, an important increase in Ca^{2+} content and elevated Ca^{2+} -ATPase activity were observed between 12 and 48 h after hepatectomy in comparison with sham-operated animals [104]. These changes appear to be followed concomitantly by an enhanced regucalcin mRNA expression [105]. Regucalcin mRNA and its translated protein have been detected [107] in hepatoma HepG2 cells.

The finding of new molecules able to activate or inhibit liver PMCA, suggests that this enzyme is modulated in a way different from that observed with erythrocyte PMCA [15, 16]. For instance, presence of a temperature-sensitive activator that is not inhibited by TFP has been postulated [15]. Moreover, an inhibitory protein of liver PMCA has been shown to mediate both Mg^{2+} and glucagon effects [16, 23]. These results raise new questions related with regulation of liver PMCA by hormones, a phenomenon that will be discussed later.

On the other hand, the protease known as calpain that activates PMCA by removing the autoinhibitory domain [76, 77], in the erythrocyte promotes cleavage of the enzyme to a 124 kD protein product that becomes maximally activated [77]. This neutral Ca^{2+} -activated protease found in rat and human liver [93] also has been involved in a wide range of pathologies [108].

Molecular forms expression of liver PMCA

PMCA is encoded by four isogenes located in different chromosomes: *pmca1* is located in chromosome 12, *pmca2* in chromosome 3, *pmca3* in chromosome X, and *pmca4* in chromosome 1 [109–112]. All genes code for a protein with a molecular weight between 127 and 135 kD [reviewed in 3, 65, 113] and contains 10 transmembrane domains connected by three cytoplasmic loops [3]. The first loop connects transmembrane domains 2 and 3 and contains the phospholipid interacting sequence. The second cytoplasmic loop connects transmembrane domains 4 and 5 and contains the catalytic site. The third intracytoplasmic loop constitutes the regulatory region of PMCA because it contains the calmodulin-binding domain, the phosphorylation sites, and the C terminus of the protein with the PDZ domain. PMCA variants are basically different in CaM binding-site length and in sequence at the carboxy end [reviewed in 3, 65, 113, 114].

A large diversity of rat and human PMCA isoforms have been described as products of alternative splicing in two main splicing sites, known as sites A and C [115, 116]. Site A is located in the vicinity of the phospholipid-responsive domain toward the 5'-end. The four encoding genes, with the ex-

ception of gene 1, produce alternative isoforms at this site. Three exons are involved in alternative splicing of *pmca2* gene, while only one is involved in *pmca3* and *pmca4* genes. Site C is very near the 3'-end of the PMCA molecule in the calmodulin (CaM) binding site. The alternative splicing process at this site is complex due to variants generated by internal donor splice sites [115, 116]. For instance, although only one exon is alternatively processed in the *pmca1* gene, it includes four internal splicing donor sequences. In the case of *pmca2* and *pmca3* two exons are involved, and in the case of *pmca4* a single exon is alternatively spliced. A *pmca4d* variant originated from an internal donor sequence in human and rat cardiac muscle, as well as in mouse C2C12 and rat Sol8 myotubes has been described [117]. Kinetic constants of several PMCA isoforms have been determined in different expression systems showing a great variability depending on the encoding gene (Table 1).

Nearly all tissues expressing PMCA proteins codified by *pmca1* and *pmca4* are considered housekeeping genes, while *pmca2* and *pmca3* distribution follows a tissue-specific pattern [66, 79, 122, 123]. Recent information obtained from null PMCA1 and PMCA4 mutants in mice, indicates a major housekeeping function for PMCA1 in comparison with PMCA4, considering that only homozygous mutants for PMCA1 produce unviable embryos [124, 125]. Housekeeping function is understood as the central and most important function for the PMCA1 form in extrusion of calcium from the cell.

Null mutants for PMCA4 are viable and female PMCA4^{-/-} mice are fertile in contrast with male PMCA4^{-/-}, which are infertile due to deficiency in sperm motility [124, 126]. In general, the observation that human PMCA1 is more abundant than PMCA4 according to corresponding mRNAs expression [66, 122, 123] or protein expression patterns [79], supports the possibility that PMCA1 indeed plays a preponderant housekeeping role.

At present, we do not possess comprehensive knowledge with regard to any pathology related with defects present in one or several PMCA genes. Nevertheless, evolutionary conservation of these Ca^{2+} -ATPases suggests that these are essential in the survival of vertebrate species [127]. Important evidence supporting the hypothesis that there is no redundancy of functions in the different PMCA isoforms has been obtained from knockout mice. It has been reported that these knockout mice, in which PMCA2 mRNA expression has been molecularly cancelled, result with severe hearing defects [128]. This physiologic effect also has been observed in mice experiencing spontaneous mutations in the PMCA2 gene involving a single substitution that exchanges Gly for Ser in the small cytoplasmic loop [129, 130] or Glu for Lys within a conserved transmembrane domain [121, 132].

Table 1. Kinetic parameters of four PMCA isoforms measured in different expression systems

PMCA isoform	1b	2a	2b	2w	2x	2z	3a	4a	4a	4b	4b	4b	4b	4b
Reference	120	71	71	118	118	118	121	119	70	119	120	70	118	71
Expression system	Sf9	COS-7	COS-7	Sf9	Sf9	Sf9	CHO	Sf9	COS-1	Sf9	Sf9	COS-1	Sf9	COS-7
Kinetic parameter														
Apparent K_m Ca^{2+} (μM)														
Without CaM	7*			~0.5*	~0.5*	~0.5*					7*		~2.0*	
Plus CaM	2*	0.09	0.06	~0.5*	~0.5*	~0.5*	0.3		0.54		2*	0.25	~0.5*	0.16
Apparent K_m ATP (nM)														
Intermediate phosphorylation	53			ND	200	ND					340		800	
Apparent K_m CaM (nM)														
Apparent CaM binding affinity	2.5	8.4	2.1	10	10	10	5–10	~650	126	~35	5.2	18	50–100	9.8
Apparent CaM binding affinity														
Ca^{2+} ATPase V_{max} (nmoles Pi/mg/min)	3*										3*			
Without CaM		ND	ND	75	80	39	210*		ND				45	
Plus CaM		ND	ND	190	210	71	700*		ND				190	
^{45}Ca -uptake (nmoles Ca^{2+} /mg/min)														
Without CaM	ND													
Plus CaM		3.67	4.21	ND	ND	ND	ND	ND			ND	ND		1.45
Without CaM		7.83	5.87	ND	ND	ND	ND	ND			ND	ND		6.15

ND = Data not determined. * Data calculated from graphs. CaM, calmodulin.

Liver pmca gene expression during normal development and pathological conditions

Human liver. Gene expression of *pmca* genes has been studied in human liver at fetal and adult stages. Spliced variants *hpmca1b*, *hpmca2*, and *hpmca4a* were detected in 20–22 week-old human male fetus by RT-PCR and by Southern blot [122]. In human adult liver, the most abundant site A splice variants correspond to *hpmca1x* (70%) and *hpmca4x* (28%), followed by *hpmca2x* and *2w*, representing $\leq 1\%$. Isoforms spliced at site C correspond to *hpmca1b*, *hpmca4b*, and *hpmca2b*, representing 70, 28, and $<2\%$, respectively [68] (Table 2). PCR amplification using degenerate mixed primers and Northern blot analysis indicated that *pmca4* was the most abundant isoform in human liver followed by *pmca1*, while the least abundant was *pmca2* [133].

Mouse and rat liver. Using *in situ* hybridization techniques, it has been shown that *pmca1* is widely expressed in mouse embryos at 9.5 days *post coitum* (*pc*), while *pmca4* was detected in fetus at 12.5 days (*pc*) with high expression in liver [123]. Recent work employing RT-PCR analysis and exploring simultaneously relative mRNA levels of *pmca1* and *pmca4* in mutants *PMCA4^{-/-}* showed *pmca1* mRNA only in adult mouse liver [124]. RT-PCR results using fetal

Table 2. *pmca* transcripts detected in rat and human liver by RT-PCR

Species	<i>pmca1</i>		<i>pmca2</i>		<i>pmca4</i>	
	SITE A	SITE C	SITE A	SITE C	SITE A	SITE C
Rat*	1x	1b	2w	2b	4x	4b
			2x		4z	4a
						4d
Human**	1x (70%)	1b (70%)	2w	2b	4x (28%)	4b (28%)
			2x			4a

*[69, 70, 133].

**[68, 122, 133].

The isoforms spliced in sites A and C are shown in decreasing order of abundance. Percentages show the most abundant isoforms detected in human liver [68].

(13 and 17 days *pc*), newborn and normal adult rat liver indicated high expression of *pmca* variants spliced at sites A and C [70]. In adult rat liver and in isolated hepatocytes, the most abundant variant was *pmca1*, and *pmca2w* was clearly detected [133]. Interestingly, *pmca4* was not detected in rat liver [131], although variant *pmca4b* has been detected by other groups [70] (Table 2). These results should be taken

with caution because it might be that relative amounts are below detection level. Moreover, certain specific conditions not well understood might be required to trigger their expression. These concepts support the relevance of PMCA in liver and open many questions related to the way *pmca* gene expression is controlled.

Kinetic experiments performed with plasma membranes isolated from regenerating liver as well as neoplastic cells of the murine hepatocarcinoma AS-30D, maintain much lower plasma membrane Ca^{2+} -ATPase activity than normal liver, while maintaining similar calcium transport. It was suggested that a more efficient mechanism to regulate calcium transport through modulation of an ATP/Pi exchange process in the catalytic cycle of the plasma membrane Ca^{2+} -ATPase present in AS-30D cells might be operating [27]. Because presence or absence of a specific isoform in the plasma membrane might explain these results, a detailed study of housekeeping the PMCA1 isoform in normal, regenerating and neoplastic liver cells in presence of the other isoforms, becomes of paramount relevance [70].

RT-PCR experiments employing AS-30D cells indicate that *pmca1x* and *pmca1b* were the most abundant variants. These isoforms were also observed in normal hepatocytes from fetal and adult rat as well as during regeneration, results that further support the housekeeping role of *pmca1* gene [68]. *pmca4a* and *pmca4d* were not detected in hepatoma AS-30D cells and *pmca4b* and *pmca2w* showed a decreased expression [70].

Proliferation-induced normal adult hepatocytes did not show alterations in *pmca1x*, *pmca1b*, and *pmca4b* expression from 2-17 days after surgery. The level of these isoforms was similar to the expression level found in growing fetal rat liver. The *pmca2w* variant as well as *pmca4x* and *pmca4z* showed lower but constant expression levels throughout liver regeneration. It is interesting to note that after 4 days of hepatectomy *pmca4a* and *pmca4d* expression was triggered; however, these splice variants are not expressed during fetal development [70].

Although at present available information as to how liver PMCA1 expression becomes regulated remains fragmented, it has been shown that hormones and agonists can induce PMCA1 expression via multiple second messenger pathways including PKC, cAMP, and Ca^{2+} as well as through participation of the *c-fos* and *jun*-proto-oncogenes [98, 99]. The second messenger pathway and sensitivity to hormones appear to be dependent on cell type. Because in the liver *pmca1* corresponds to the predominant isoform, future work is needed to determine how expression of this gene and its splicing pattern is controlled. Nonetheless, it is known that prolonged hormone treatments induce *pmca* mRNA [98] accumulation, where characteristics of the PMCA promoter correspond to a housekeeping gene [134].

At protein level, PMCA1 is the sole isoform detected to date in adult rat liver by Western blot [81]. Why *pmca2* and *pmca4* splice transcripts variants seem not to evolve to mature mRNAs? Which mechanisms control their translation? Are these splice variants expressed by non-parenchymal cells? These are open questions for which answers are still needed.

Hormonal regulation of liver PMCA

PMCA regulation has been studied by different groups based on hormone-dependent free Ca^{2+} concentration changes in liver cells [18, 23, 31, 135–144]. Vasopressin added directly to liver plasma membrane vesicles does not modify Ca^{2+} -ATPase activity [31]. However, when the liver is perfused with vasopressin at different concentrations from 10^{-10} to 10^{-7} M, gradual inhibition of ^{45}Ca -uptake is induced [31]. This effect is also induced by perfusion with other Ca^{2+} -mobilizing hormones such as 10^{-5} M epinephrine and 10^{-7} M angiotensin II, but not by 10^{-7} M glucagon [31]. Isolated hepatocytes incubated with vasopressin at nanomolar concentration also show a decrease in plasma membrane Ca^{2+} -ATPase activity, whereas insulin and 10^{-10} M glucagon do not modify enzyme activity [136]. Later, it was found that the C-proteolytic product of glucagon, glucagon-(19–29) also known as miniglucagon [137], corresponds to a better inhibitor than glucagon itself [138, 139] (Fig. 1). Nanomolar concentrations of miniglucagon inhibit Ca^{2+} -ATPase with a mechanism associated with heterotrimeric G proteins without adenylate cyclase activation [139–141]. A cholera toxin-sensitive $\text{G}\alpha_s$ protein has been shown as mediator of the inhibition [142]. Furthermore, a G_s protein also mediates the inhibitory effect of human PTH, calcitonin [143], and endothelin B [144] on rat liver PMCA. These results support early work related with regulation of liver Ca^{2+} -ATPase by membrane-associated proteins [15, 16, 18] (Fig. 1).

Agonist-induced free Ca^{2+} transients and PMCA in hepatocytes

Changes in hepatocyte intracellular Ca^{2+} levels induced by an agonist-receptor complex signaling through the phosphoinositide pathway, together with the spatial and temporal organization of cytoplasmic calcium signals, have been studied in isolated hepatocytes as well as in whole liver [145–147]. Vasopressin, angiotensin II, noradrenaline [148] as well as glucagon, ADP and ATP [149–152] promote a train of spikes from basal 100–150 nM Ca^{2+} to peak 600 nM free Ca^{2+} concentration. Time-course of these Ca^{2+} transients is agonist-specific, i.e. vasopressin induces spikes of 10-sec duration, whereas ATP induces oscillations of up to 90-sec duration using isolated hepatocytes. Recovery rate of

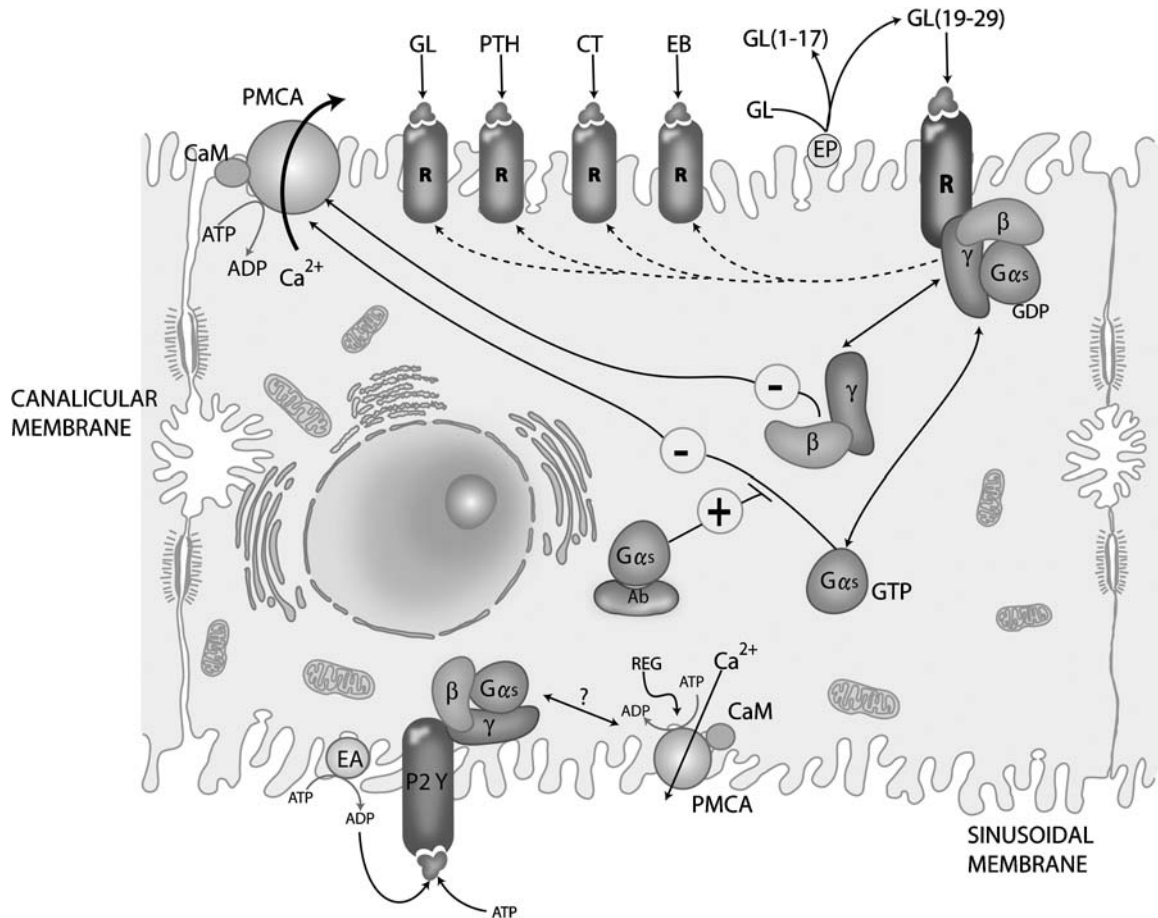


Fig. 1. Regulation of plasma membrane Ca²⁺-ATPase (PMCA) activity in the hepatocyte. Glucagon (GL) and its product miniglucagon (GL 19–29) generated by an intramembrane endonucleopeptidase (EP) that cleaves specifically between Arg¹⁷ and Arg¹⁸, have been involved in regulation of hepatocyte PMCA. Glucagon, miniglucagon, parathyroid hormone (PTH), endothelin B (EB), and calcitonin (CT), have been shown to inhibit liver PMCA activity through G protein action, independently of adenyl cyclase activation. GTP promotes dissociation of the heterotrimeric G protein, releasing subunits α and $\beta\gamma$ that inhibit PMCA activity. Antibodies antiG α_s (G α_s Ab) restore PMCA activity. The presence of an ecto-ATPase (EA) in the plasma membrane of the sinusoidal domain, releases ADP from ATP and becomes a ligand for P2Y purinergic receptors known to interact with G proteins, that in turn modulate PMCA activity. The role of G proteins upon cytosolic Ca²⁺-binding proteins, such as calmodulin (CaM) and regucalcin (REG) known to modulate PMCA activity, represent also an important regulatory mechanism of PMCA.

individual Ca²⁺ transients is clearly different for each agonist but transient frequency is dependent on agonist concentration as well as on extracellular calcium concentration [145, 146]. Heterotrimeric G proteins of the G α_q class couple the plasma membrane agonist-receptor complex to isoforms of phospholipase C (PLC) to activate production of inositol-1, 4,5-triphosphate (IP₃) and diacylglycerol (DAG) through hydrolysis of phosphatidylinositol 4,5-bisphosphate. IP₃ binds to its receptor in the endoplasmic reticulum membrane and activates release of calcium to the cytosol [153] (Fig. 1). This flux together with Ca²⁺ entry through the plasma membrane [154] explain the first phase of Ca²⁺ transients. The falling phase of the spike involves re-uptake of calcium by SERCA and/or by Ca²⁺ being pumped out of the hepa-

toocyte by PMCA [155]. It is noteworthy that the Na⁺/Ca²⁺ (NCX) exchanger appears to play only a minor role in regulation of Ca²⁺ in rat hepatocytes [156,157]; however, some evidence points to a possible role for this exchanger under oxidative stress in human hepatoma cells (HepG2) [158].

A 50% PMCA inhibition carried out by miniglucagon in rat hepatocytes [159], shows Ca²⁺ transients elicited by ATP or by phenylephrine. The recovery phase of these agonist-induced Ca²⁺-transients was not affected by PMCA activity inhibition. Moreover, PMCA inhibition supported prolonged generation of Ca²⁺ oscillations in absence of extracellular calcium [159]. These results suggest that hormone-induced PMCA inhibition could increase Ca²⁺ mobilization elicited by different agonists in hepatocytes

[31]. Because frequency of Ca^{2+} oscillations also depends on extracellular level of calcium and on rate of Ca^{2+} influx [160, 161], an interaction appears possible between hepatocyte extracellular calcium-sensing receptor [162] and proteins controlling PMCA activity.

Glycogen metabolism and bile secretion appear to be under control of these Ca^{2+} oscillations [135, 162]. Agonist-induced Ca^{2+} waves originate from a specific site in the

apical region, where IP_3R is localized [43], and propagate to basolateral region and nucleus of hepatocytes [163–167]. Ca^{2+} oscillations are propagated to neighbor hepatocytes and eventually to whole liver by diffusion of IP_3 and/or Ca^{2+} itself through gap-junctions and also to the nucleus of hepatocytes [164, 168, 169]. Activation of IP_3R and ryanodine receptors (RyR) mediates propagation of the Ca^{2+} signal (Fig. 2). Based on pharmacologic and functional evidence

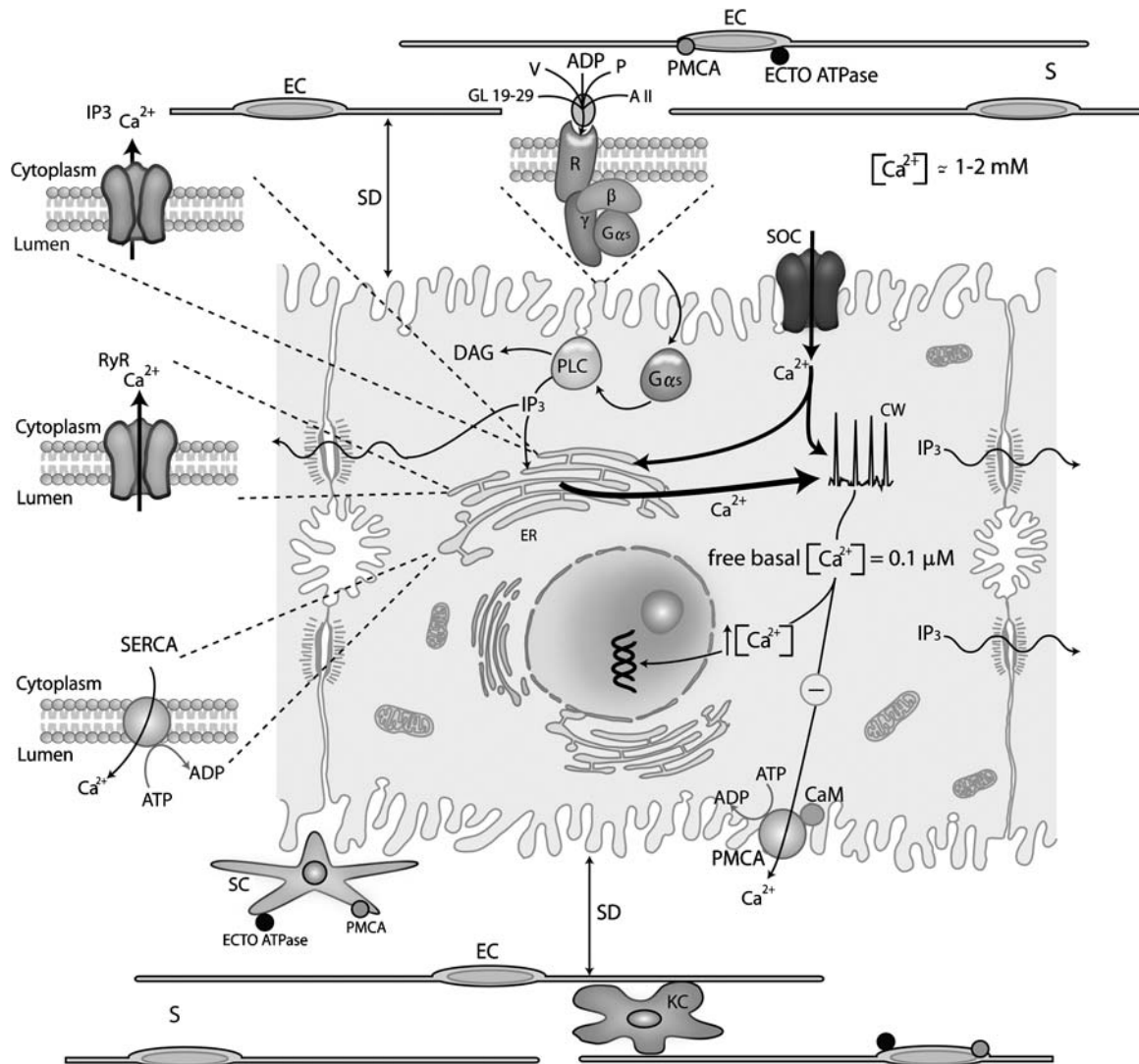


Fig. 2. Calcium homeostasis in the hepatocyte. Vasopressin (V), angiotensin II (AII), phenylephrine (P), miniglucagon (GL 19-29), and ADP induce Ca^{2+} transients by a common mechanism involving inositol-1,4,5-triphosphate (IP_3). The basal Ca^{2+} level ≈ 100 nM, increases up to 700 nM after release of Ca^{2+} from the endoplasmic reticulum (ER). Focal activation of IP_3 receptors (IP_3R) localized in the ER induces efflux of Ca^{2+} from ER stores. Ryanodine receptors (RyR) localized in ER membranes also release Ca^{2+} to the cytosol. A Ca^{2+} concentration decrease in the ER activates plasma membrane store-operated Ca^{2+} channels (SOCs), that in turn activates the sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA). Propagation of the Ca^{2+} signal within a single hepatocyte and towards neighbor hepatocytes through gap junctions, is mainly by diffusion of IP_3 . An increase in free basal cytoplasmic Ca^{2+} -concentration stimulates a Ca^{2+} flux into the nucleus, inducing changes in gene expression and alternative splicing. Kupffer cells (KC) releasing several important growth factors together with stellate cells (SC), play also an important role in hepatocyte calcium homeostasis. Endothelial cells (EC), Space of Disse (SD), Sinusoids (S), and Ca^{2+} -waves (CW).

it seems that non-muscle tissues such as liver might present RyR [170, 171]. Heterogeneity in agonist receptor density as well as in hepatocyte sensitivity (pericentral hepatocytes are more sensitive to vasopressin than periportal hepatocytes, but the opposite is true for ATP), leads to directional activation of Ca^{2+} -dependent processes [165–167]. Because PMCA plays a major role in regulation of cytosolic Ca^{2+} levels, a gradient of PMCA activity could be associated with the direction of Ca^{2+} -waves. Whether these Ca^{2+} waves regulate PMCA gene expression or splice variant isoforms formation deserves future work [169, 172] (Fig. 2).

On the other hand, cytoplasmic Ca^{2+} concentration is increased by 2,5 di-(tertbutyl)-1,4-benzohydroquinone (tBuBHQ) inducing release of Ca^{2+} from ER without IP3R participation [173]. Hepatocytes exposed to vasopressin, angiotensin II or ATP, show increase in efflux of Ca^{2+} and a rapid return to basal Ca^{2+} levels takes place [173–175]. This effect is also induced by NaF and AlCl_3 , indicating participation of G-proteins in calcium efflux pathway stimulation [176, 177]. Whether adenosine [178] and bile acids [179, 180], which induce mobilization of Ca^{2+} from ER without IP₃R participation stimulates PMCA activity, remains to be defined.

The results presented previously further confirm the role of PMCA in control of basal free Ca^{2+} concentration and also demonstrate PMCA interaction with G protein-coupled receptors. Since hepatocyte PMCA activity is negatively regulated by G proteins, it appears very likely that specific G protein signaling regulators involved in control of signal termination and in the delay observed between Ca^{2+} oscillations [181] are also involved in PMCA regulation. Subtype-selective G protein coupling to different effectors [182, 183] including PMCA, may also provide new clues to understanding PMCA regulation.

Perspectives

Many functions in liver are under regulation through calcium-mediated processes. For instance, glucose production responds to a Ca^{2+} increase induced by glucagon, as well as processes involved in bile secretion, such as vesicular trafficking and canalicular exocytosis. Likewise, Ca^{2+} presents an important role in cell growth regulation and consequently in events related with apoptotic and necrotic death [184]. Due to the importance of calcium homeostasis in liver physiology, precise control of its intracellular concentration is critical for survival and normal functioning of the hepatocyte [185] (Fig. 2). To achieve normal calcium homeostasis, PMCA actively works in coordination with the NCX located in plasma membrane as well as intracellular systems located in sarcoendoplasmic reticulum, mitochondria, and nucleus. Among these systems, PMCA presents the highest affinity

for Ca^{2+} and therefore the best capacity to respond to critical cytosolic Ca^{2+} fluctuations. It is interesting to note that recent information emphasizes that PMCA activity is sufficient to maintain Ca^{2+} -homeostasis in cardiac specific NCX knockout mice [186]. In plasma membranes, the role of the store-operated Ca^{2+} channels (SOCs) is also important to restore the intracellular pool after massive release of calcium from the endoplasmic reticulum [147, 187, 188]. Non-parenchymal cells such as endothelial cells, Kupffer cells, lymphocytes, and stellate cells should also be taken into consideration, since they most probably contribute importantly to maintenance of calcium homeostasis in liver. Since the reason for the existence of such a large number of PMCA isoforms is still not well-understood, identification of isoform-specific interactions with partner proteins might provide a clue [189].

A question that still remains to be answered is related with the fact that we do not know whether *pmca2* and *pmca4* transcripts are efficiently translated into proteins in liver, or whether the amount of protein is so low that these transcripts can not be quantified and are therefore difficult to measure. The signaling cascade controlling these genes and the detailed study of their promoter regions also remain to be defined. Moreover, the design of specific inhibitors of Ca^{2+} -ATPase will aid in extending our understanding of the properties of these P-type ATPases. In this respect, the peptide caloxin 3A1 was described recently as an efficient inhibitor of plasma membrane Ca^{2+} -ATPase by means of interaction with the third extracellular region of PMCA (PED₃) [190].

Although based on evidence, the answer for our initial question might be yes, a systematic study of the different PMCA isoforms expressed in normal liver as well as in regenerating and neoplastic liver cells, will undoubtedly help to reveal hormone-related control mechanisms associated to calcium homeostasis in this tissue. New strategies directed toward definition of the correlation between isoform-structure and function, will also contribute to solve the mystery within which for many years the liver Ca^{2+} -ATPase has been shrouded.

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