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Review

Plasma membrane Ca^{2+}-ATPase in excitable and nonexcitable cells★✪

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There is a significant number of data confirming that the maintenance of calcium homeostasis in a living cell is a complex, multiregulated process. Calcium efflux from excitable cells (i.e., neurons) occurs through two main systems — an electrochemically driven Na^+/Ca^{2+} exchanger with a low Ca^{2+} affinity ($K_{0.5}$ = 10–15 μM), and a plasmalemmal, specific Ca^{2+}-ATPase, with a high Ca^{2+} affinity ($K_{0.5}$ < 0.5–1 μM), whereas in nonexcitable cells (i.e., erythrocytes) the calcium pump is the sole system responsible for the extrusion of calcium ions. The plasma membrane Ca^{2+}-ATPase (PMCA) is a ubiquitously expressed protein, and more than 26 transcripts of four PMCA genes are distributed in a tissue specific manner. Differences in the structure and localization of PMCA variants are thought to correlate with specific regulatory properties and may have consequences for proper cellular Ca^{2+} signaling. The regulatory mechanisms of calcium pump activity have been studied extensively, resulting in a new view of the functioning of this important molecule in the membranes.

Calcium ions serve as the main second messengers in all cell types, and in the cytosol free Ca^{2+} concentration is maintained at a very low level (50–150 nM). Fluctuations in

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Abbreviations: PMCA, plasma membrane Ca^{2+}-ATPase; CaM, calmodulin; PMA, phorbol 12-myristate 13-acetate; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; PKA, protein kinase A; PKC, protein kinase C; P-Ser, phosphoserine; P-Thr, phosphothreonine; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate.

intracellular Ca^{2+} level are essential elements for normal cellular activities which are closely connected with the development of cells, mitotic activity, immune response, muscle contraction, endo- and exocytosis, or modulation of neuronal cells processes [1, 2]. The precise regulation of the Ca^{2+} homeostasis in cells is a result of the concerted functioning of transporters located in the plasma membrane, systems operating in cell organelles, i.e., endo/sarcoplasmic reticulum, mitochondrium, nucleus, and calcium binding proteins. Calcium efflux from excitable cells occurs through two main systems, an electrochemically driven Na^+/Ca^{2+} exchanger with a low Ca^{2+} affinity, and a plasmalemmal Ca^{2+}-ATPase, with a high Ca^{2+} affinity [3]. The capacity of the Na^+/Ca^{2+} exchanger to pump out calcium ions is more than 10 times greater when compared to plasma membrane Ca^{2+}-ATPase. In nonexcitable cells the calcium pump is the sole system responsible for the extrusion of calcium ions outside the cells. Thus, plasma membrane Ca^{2+}-ATPase is considered a fine tuner of cytosolic calcium ion concentration, and the important role of this enzyme is also reflected in its isoform-specific ubiquitous expression among different cell types.

Typically, the plasma membrane Ca^{2+}-ATPase (PMCA) pump represents a minor fraction (about 0.1%) of the erythrocyte proteins, but in synaptosomes Ca^{2+}-ATPase is present at a higher level than in erythrocyte ghosts [4]. Calcium pump belongs to the P-type ATPase family the members of which are able to utilize the energy of ATP to transport ions against their electrochemical gradients across membranes. The Ca^{2+}-ATPase reaction cycle involves the sequential formation and degradation of phosphorylated intermediates (aspartylphosphate). The enzyme that can exist in two different states (E_1 and E_2) with different affinities for Ca^{2+} undergoes a conformational change during the transport of a Ca^{2+} ion. Experimental data have shown that the pump operates as an electrogenic Ca^{2+}/H^+ exchanger with a 1:1 stoichiometry [5].

Several modes of Ca^{2+}-ATPase activation have been reported, including stimulation by a naturally existing activator — calmodulin, acidic phospholipids, long chain polyunsaturated fatty acids, proteolytic treatment by calpain, protein G $\beta\gamma$ subunits, oligomerization, and more recently proved, phosphorylation by protein kinases (for review see [6, 7]). The studies reviewed in this paper describe some modulatory mechanisms that could be of physiological and pathological importance.

PLASMA MEMBRANE Ca^{2+}-ATPase ISOFORMS IN EXCITABLE AND NONEXCITABLE CELLS

In a number of cell types Ca^{2+}-ATPase has been shown to be associated with membrane structures named caveolae that are thought to be involved in multiple signal transduction events at the cell surface [8]. These caveolae represent a dynamic cell surface-membrane system. Moreover, some cellular elements of calcium-dependent machinery i.e., IP_3 receptors, calmodulin and nitric oxide synthase are concentrated in caveolae. Thus, the specific PMCA localization appears to be primarily related to the involvement of Ca^{2+} signaling in the regulation of transduction and adaptation mechanisms.

As found with other ion pumps, Ca^{2+}-ATPases form a multigene family. Plasma membrane calcium pump isoforms are encoded by 4 different genes named *pmca 1*, *pmca 2*, *pmca 3* and *pmca 4*, and due to alternative mRNA splicing more than 26 variants can exist (Table 1) [8–10]. Based on functional studies it has been shown that these isoforms and variants differ in their regulatory properties. In human chromosomal localization of the *pmca* genes has been described (Table 2) [11]. Among the best characterized

members of the P-type ATPase family is the calcium pump of erythrocyte membrane. This family represents a mixture of isoforms

Table 1. Plasma membrane Ca^{2+}-ATPase isoforms [8–10]

Gen	Number of variants	Number of amino acids	M_r
pmca 1	5	1176–1258	129200–138800
pmca 2	8	1154–1243	127500–136800
pmca 3	9	1117–1230	123500–136000
pmca 4	6	1170–1205	129400–133900

PMCA 1 and PMCA 4 which are expressed in practically all tissues, while PMCA 2 and 3 have been shown to occur in highly specialized tissues, especially in the brain and heart [3, 7, 12]. It is noteworthy that the brain contains up to 10 times more PMCA than nonexcitable cells [10].

During the last years, using molecular biology techniques, *in situ* hybridization and isoform specific monoclonal antibodies it has been demonstrated that mRNAs and PMCA proteins were nonuniformly distributed in different regions of human, mouse, rat, pig and gerbil brains [13–19]. The amounts of PMCA 2 and PMCA 3 were similar to those of PMCA 1 and PMCA 4, however, there were some subtle differences among brain areas. Recently, it has been reported that PMCA pump is present in mammalian glial cells [20]. The amounts of PMCA 1 and PMCA 4 isoforms in astrocytes were comparable to those found in neurons, whereas the amount of PMCA 2 was lower. These data strongly indicate that each brain region may utilize a unique combination of isoforms to maintain intracellular Ca^{2+} control. Moreover, the expression of particular PMCA isoform is regulated during development [19, 21, 22]. There is a direct evidence for the involvement of calcineurin in the control of neuronal transcription of the PMCA 4 isoform [23]. The existence of multiple isoforms has also been described for all excitable and nonexcitable cells examined so far. In addition, in several types of cultured cells PMCA mRNA was induced by various agonists *via* multiple second messenger pathways [24].

Table 2. Chromosomal localization of human PMCA genes [11]

Gen	Chromosome	Locus
pmca 1	12	q 21–q 23
pmca 2	3	p 26–p 25
pmca 3	X	q 28
pmca 4	1	q 25–q 32

PHOSPHORYLATION PROCESSES IN THE REGULATION OF CALCIUM PUMP ACTIVITY

Now it is well-established that nearly all events of cell functioning are regulated by reversible protein phosphorylation. The genomes of higher eukaryotes encode approximately 2000 and 1000 protein kinases and phosphatases genes, respectively, corresponding to 3% of the genome [25]. Serine and threonine are the major amino-acid residues phosphorylated in cells. In contrast, the level of tyrosine phosphorylation is below 0.1% of serine/threonine phosphorylation [26]. Reversible phosphorylation of serine and threonine has been demonstrated to regulate the activity of a number of membrane enzymes.

Calcium pump has also been identified as a target for protein kinases actions. The C-terminal part of Ca^{2+}-ATPase is a multifunctional regulatory region, and contains an

autoinhibitory domain with a high affinity calmodulin binding site, acidic phospholipids-binding site, and sequences that are potent targets for phosphorylation by protein kinases [4]. In PMCA variants the sequences phosphorylated by protein kinases are not conserved and phosphorylation may differently regulate the calcium pump activity [6]. Although many details remain unclear, the importance of this mechanism seems to be unquestionable.

The first report dealing with this subject described the activation of erythrocyte calcium pump by protein kinase A [27]. Later it has been found that PKA phosphorylated the serine residues located in the calmodulin-binding domain of the PMCA 1 isoform [28]. Although this isoform, as well as its spliced variants, are thought to be a house-keeping form of the enzyme in many cells, the transcripts encoding a potentially PKA-insensitive PMCA 1 isoform have also been detected, particularly in the brain [29]. PKC-mediated phosphorylation has been demonstrated in purified red blood cell Ca^{2+}-ATPase, in human neutrophils, intact human platelets, cultured aortic endothelial cells, vascular smooth muscle cells, and murine lymphocytes [30–35]. PKC phosphorylated a threonine residue, and recent studies have demonstrated that at least one serine residue located carboxy-terminally to the CaM-binding domain was also the substrate for this kinase. A study performed with isoform PMCA 2 and PMCA 3 variants overexpressed in COS cells revealed that PKC regulated their activity in different ways [36]. Little or no phosphorylation by PKC was detected in PMCA 2b and PMCA 3b, whereas PMCA 2a and 3a variants were phosphorylated without increasing their Ca^{2+} transport activity. It is noteworthy that phosphorylation prevented stimulation of Ca^{2+}-ATPase by calmodulin (CaM). Phosphorylation of PMCA 4a was blocked when CaM was bound to the enzyme, but phosphorylation in the absence of CaM did not eliminate either binding or further activation of the calcium pump by CaM [37]. In conclusion, in COS cell membranes, PMCA variants: 2a, 3a, 4a and 4b are phosphorylated by PKC, but only PMCA 4b is activated by this process [6, 38].

Up to now, only partial data have been published on possible regulation of the calcium pump by phosphorylation in the nervous tissue [39]. Recently, it has been reported that the PKC inhibitor, sphingosine, is a weak inhibitor of the synaptosomal but an effective inhibitor of the leukocyte membrane Ca^{2+}-ATPase activity, and according to Grosman these differences could depend on the origin of the membranes [40]. Our previous study revealed that *in vitro* Ca^{2+}-ATPase activity in rat cortical and cerebellar synaptosomal membranes increased in the presence of PKA and PKC activators, i.e., cAMP and phorbol derivatives, respectively [41]. Okadaic acid, a specific inhibitor of protein phosphatases PP1 and PP2A, brought about further enhancement in the calcium pump activity. It is noteworthy that the intensity of this regulation differed in a brain region-dependent manner. We showed that calcium pump purified from rat cortical, cerebellar and hippocampal synaptosomal membranes contained P-Ser and P-Thr, thus the phosphorylation of Ca^{2+}-ATPase appears to be a physiological phenomenon, however, the nature of protein kinases that were responsible for this process *in vivo* remains unknown [42]. Moreover, purified Ca^{2+}-ATPase was a substrate for phosphorylation *in vitro* by protein kinases A and C. It should be kept in mind that the individual isoforms and variants are expressed at different abundances in living cells, and that the state of phosphorylation of Ca^{2+}-ATPase results from the concerted action of cellular protein kinases and phosphatases, modulated by several second messenger systems.

DEPHOSPHORYLATION PROCESSES IN THE REGULATION OF CALCIUM PUMP ACTIVITY

In contrast to phosphorylation, limited data are available on specific Ca^{2+}-ATPase dephosphorylation. The protein Ser/Thr phosphatases PP1, PP2A and PP2B account for the majority of the phosphatase activity *in vivo,* and are involved in multiple cellular functions [25]. The effect of PP2A-mediated dephosphorylation on calcium pump activity has only been observed in the membrane-inserted and purified erythrocyte enzyme [43]. The nervous tissue is particularly enriched both in protein kinases and protein phosphatases, with differing substrate specificities [26]. Protein phosphatases participate in a variety of signaling pathways, including the most potent Ca^{2+}-induced phenomena that modulate the neuronal cell activity. PP1 is present in membrane fractions and synaptic junction, and is also associated with neurofilaments [25]. The heterotrimeric PP2A is expressed in a cell- and tissue-specific fashion, and a neural function has been inferred from the targeting of PP2A to specific intracellular location [25, 26].

We have demonstrated that, under an *in vitro* assay, PP1 and PP2A decreased the activity of purified cortical and cerebellar calcium pump [44]. This provides further support for *in vivo* phosphorylation of Ca^{2+}-ATPase in neuronal cells. Moreover, the native enzyme appeared to be a substrate for PP1 and PP2A *in vitro*. The decreased activity of dephosphorylated Ca^{2+}-ATPase was associated with its enhanced potency for stimulation by calmodulin, and it could suggest that both activatory mechanisms, i.e., phosphorylation and CaM-stimulation, are competitive under the *in vitro* assays. The regulation of calcium pump activity appears to be a more complex phenomenon, because recently it has been reported that protein phosphatases PP1 and PP2A reversibly inhibit PKCα activity [45]. Thus, these phosphatases could directly and/or indirectly (*via* PKC) regulate the calcium pump activity in cells.

Taken together, the complex effects of the reversible phosphorylation result from cross-activation of calcium pump by different regulatory mechanisms which are strongly dependent on the cell type and PMCA isoforms presence.

MULTISTEP ACTION OF NEUROACTIVE STEROIDS ON PLASMA MEMBRANE Ca^{2+}-ATPase

During the last years there has been an increasing number of data confirming that brain is a site of extensive synthesis and metabolism of steroid hormones', and that the accumulation of steroids appears to be, at least in part, independent of adrenal and gonadal sources [46]. Beside their actions at the transcriptional level, steroids may act on nerve cells *via* plasma membrane receptors. Biochemical and electrophysiological experiments have demonstrated that neuroactive steroids are potent allosteric modulators of $GABA_A$, nicotinic, muscarinic, NMDA receptors, and some receptors coupled to G-proteins [47–50].

One of the recent insights concerning the regulatory mechanism of brain Ca^{2+}-ATPase function is the direct and indirect influence of neuroactive hormones. In synaptosomal membranes of dog brain, testosterone has been observed to increase the activity of Ca^{2+}-stimulated ATPase, whereas progesterone had an opposite effect [51]. In rat brain neuronal membrane preparations an increase of the activity of Ca^{2+}-ATPase was demonstrated after a short-time incubation with physiologically relevant concentrations of pregnenolone sulfate and 17-β-estradiol [52]. Later experiments performed on purified rat cortical Ca^{2+}-ATPase showed that at biologically relevant concentrations (pM to nM) the neuroactive steroids: 17-β-estradiol, testosterone, pregnenolone sulfate and dehydro-

epiandrosterone sulfate, were responsible for the direct stimulation of Ca^{2+}-ATPase hydrolytic activity [53]. All neuroactive steroids examined decreased also calmodulin stimulation of Ca^{2+}-ATPase. More importantly, they were more effective than CaM in the activation of purified Ca^{2+}-ATPase. Although the precise mechanism of these regulations remains unknown, the CaM-binding domain of Ca^{2+}-ATPase is assumed to be a primary site of steroids action. These observations may have physiological consequences, because local steroid synthesis could allow permanent, Ca^{2+}-independent regulation of Ca^{2+}-ATPase activity in neuronal plasma membranes, whereas the calmodulin binding is a Ca^{2+}-dependent process. However, a better understanding of the molecular mechanisms of the non-genomic Ca^{2+}-ATPase regulation by steroid hormones needs further study.

MEMBRANE EFFECTS OF REACTIVE OXYGEN SPECIES ON CELL IONS HOMEOSTASIS

Under normoxic conditions a number of biochemical reactions generate reactive oxygen species (ROS) which are neutralized by the antioxidant defense systems. The excessive production of ROS results in enhanced oxidation of cellular biomolecules, including lipids, DNA, proteins and amino acids [54]. Recent studies have indicated that the ROS-dependent oxidative modification of biological membranes is particularly more pronounced during hypoxia-ischemia cell injury [55]. Using different models it has been documented that increased reactive oxygen species production could propagate an oxidative cascade in cells. For example, a hypoxic tissue underwent lipid peroxidation 5 times faster than a normoxic tissue, and the level of thiobarbituric acid reactive substances (TBARS) was three times higher in the hypoxic tissue [56]. Peroxidation of membrane lipids results in changes in fluidity and permeability, which can also affect functioning of membrane proteins. Structural rearrangement of membrane proteins is frequently correlated with increased amounts of disulfide bonds, carbonyl groups, and nitro- or aminotyrosine formation [57, 58].

Several essential proteins are known to be particularly sensitive to oxidative modifications, including ion channels, Na^+, K^+-ATPase, and glucose and glutamate transporters (for a review see [59]). It has been shown that oxidized proteins are often functionally inactive, and are more susceptible to proteolytic cleavage. Several lines of evidence suggest that plasma membrane Ca^{2+}-ATPase is among the proteins which are a target for reactive oxygen species. Ca^{2+}-ATPase shows a diminished activity following ascorbate/iron induced oxidation, and a similar effect has been observed after Fe^{2+}/H_2O_2 incubation [60, 61]. Oxidative stress can increase intracellular Ca^{2+}concentration and trigger the series of events due to the generation of reactive oxygen species. One of the most profound consequences is the activation of nitric oxide synthase, resulting in the production of NO [59]. A direct reaction of NO and O_2^- produces peroxynitrite that can exert pathological consequences in the cell. $ONOO^-$ is a powerful oxidant, which can react with unsaturated fatty acids or amino-acid residues [62]. Recently, it has been demonstrated that $ONOO^-$ crosses cell membranes at a rate significantly higher than that of its decomposition pathways [63]. Erythrocyte membranes exposed to peroxynitrite have shown aggregation and nitration of proteins, changes in protein organization, and inactivation of the Ca^{2+}-ATPase activity [57].

We have recently shown that purified erythrocyte plasma membrane Ca^{2+}-ATPase is directly degraded by peroxynitrite, and a similar degradation pattern has been detected in erythrocyte membranes of asphyxiated newborns [64]. The Mg^{2+}-dependent Ca^{2+}-ATPase activity in erythrocyte ghosts of asphyxiated

newborns was diminished by 50% when compared with healthy newborn infants. Moreover, the activity of Ca^{2+}-ATPase in asphyxiated membranes was stimulated by CaM to a lesser degree than in normal membranes. The decreased activity of the enzyme was correlated with aggregation and degradation of the calcium pump molecules.

Under physiological conditions due to reactive oxygen species action protein and amino-acid peroxides may also be formed. They are relatively stable species and could decrease the membrane -SH group content. Amino-acid peroxides have been shown to inhibit Na^+/K^+-ATPase and Ca^{2+}-ATPase of erythrocyte membrane, as well as being responsible for the oxidation of hemoglobin to methemoglobin [65].

Disturbances in calcium homeostasis play a particularly important role in brain damage, and in a number of processes that have been implicated in the Ca^{2+} related pathogenesis including brain aging, ischemia/anoxia, oxidative stress, and Alzheimer's or Parkinson's diseases (for a review see [54]). In synaptic plasma membranes of rat brain after exposure to peroxyl radicals, H_2O_2 and $ONOO^-$, significant cross-linking of PMCA molecules has been detected, as well as diminished calcium pump activity [66]. In another study the Ca^{2+}-dependent ATPase activity in synaptosomal plasma membranes was significantly depressed following peroxidation of membrane lipids [61]. Thus, the decreased activity of calcium pump after ROS exposure could result from both the alteration of the lipid environment and direct modification of the polypeptide chain.

CONCLUSION

In addition to a number of well-documented regulatory mechanisms of plasma membrane Ca^{2+}-ATPase, the action of protein kinases and phosphatases, modulatory effect of some neuroactive steroids, and modification of calcium pump function by reactive oxygen species appear to be important from the physiological and pathological points of view. These regulatory mechanisms could be specifically coupled to distinct signaling pathways in excitable and nonexcitable cells. Moreover, interdependence between different regulatory modes of plasma membrane calcium pump may be essential for the complex Ca^{2+} signaling.

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