Cell Calcium Extrusion Systems and their Role in Epileptogenesis

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Abstract: The precise control for maintenance of a normal intracellular calcium concentration in eukaryote cells is accomplished by several systems located at the plasma membrane, as well as several internal membrane systems. Neurons are especially sensitive to changes in these control systems, since when fail and calcium homeostasis disturbed, the cell's metabolism is immediately modified and a pathological condition emerges. Such a condition has been associated with epileptogenesis, and especially to those mechanisms associated to calcium entrance or ON mechanisms. On the other hand, calcium extrusion mechanisms or OFF mechanisms, have been investigated to a lesser extent and therefore remain much less understood. Here, we present a review of these calcium extrusion systems located at the plasma membrane considered to be critical in the process of epileptogenesis; first of all the plasma membrane calcium ATPase (PMCA) as the catalytic moiety of the enzyme that moves calcium outwards in an energy-dependent fashion, and the Na^+/Ca^{2+} exchanger (NCX) coupled to the (Na^+/K^+)-ATPase. Based on present knowledge considering the wide range of isoforms found for PMCA and NCX and their specific kinetic characteristics, a hypothesis for their participation on the OFF mechanisms related to the genesis of epilepsy is discussed.

Keywords: Epilepsy, epileptogenesis, calcium regulation, PMCA, NCX, calcium extrusion proteins.

1. INTRODUCTION

Epilepsy can be defined as a chronic illness of diverse etiology characterized by recurrent crises due to an excessive and synchronic burden of cerebral neurons, eventually associated with diverse clinical and paraclinical manifestations. Epilepsy is a common pathology; World Health Organization (WHO) statistics revealed in the year of 2001 a prevalence of 8.2 per 1,000 individuals in developed countries and 10 per 1,000 in developing countries. During the same year, incidence in developed countries was 50 per 100,000 individuals in the general population, and 100 per 100,000 in developing countries. The analysis we have performed in the present study is related to the 50% of these patients that present by diverse external causes an acquired epilepsy [1]. One very important period of epilepsy comprises epileptogenesis, i.e., the period in which epilepsy is developed, which can be considered the period between the lesion and the appearance of clinical manifestations. Epileptogenesis includes all phenomena that induce normal cells to discharge abnormally, which when repeated in a continuous fashion, produce an epileptic focus. For these phenomena to be expressed in cells, a change is required in the majority of systems controlling neuronal excitability and inhibitory processes. Such phenomena allow an exaggerated abnormal discharge of neurons provoking hyperexcitability in the long term. During the period of epileptogenesis, there also appear aberrant interconnections that promote neuronal synchronization with the consequent clinical manifestations [1].

Calcium is an important regulator in many metabolic pathways, as well as a second messenger; therefore, its intracellular concentration is regulated precisely. Transitory elevations in calcium are created during diverse physiological processes such as synaptic transmission, long-term potentiation in the learning process, cellular growth and differentiation, cytoskeleton maintenance, and genetic expression. Intracellular calcium is maintained between 50 and 200 nM, which represents four orders of magnitude beneath its extracellular concentration [2,3]. When there is a considerable and irreversible increase in the intracellular concentration of calcium (glutamate-associated citotoxicity), diverse mechanisms of cellular death are triggered. At the intermediate point between normal regulation of calcium inflow and intracellular calcium cytotoxicity-related cell death, a nonlethal, prolonged, and irreversible intracellular calcium increase occurs, triggering a series of abnormal plastic changes termed epileptogenesis. After a lesion takes place at the epicenter, these changes are severe and lead to cell death. Nonetheless, cells surrounding the epicenter experience less severe changes and form the substrate for the development of epilepsy [4]. For an adequate maintenance of calcium levels, cells are provided with specialized mechanisms for increasing cytoplasmic calcium concentration termed ON mechanisms, while those devoted to extrude calcium are considered the OFF mechanisms. A massive inflow of calcium during epileptogenesis involves several important consequences, such as changes in neuronal excitability [5], apoptosis induction, and fiber reorganization known as sprouting. The sprouting phenomenon possesses relevance because it promotes an important increase of excitatory circuits, mainly those of the recurrent type [1]. Although calcium inflow mechanisms have been extensively studied and their implication in epilepsy is well known, calcium extrusion processes are less understood. In this review, therefore, we focus on the investigation carried out studying mainly the calcium

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extrusion mechanisms, specifically those located at the plasma membrane.

In hippocampus-cultured cells in which epileptiform activity is provoked by lowering magnesium in the perfusion medium, glutamate was added to the extracellular medium to measure glutamate-mediated calcium rises; chronic elevated basal intracellular calcium levels were found [6]. In order to observe the participation of ON mechanisms (in the induction phase of epilepsy) during exposure to glutamate, N-methyl-D-aspartate (NMDA) receptors and voltage-dependent calcium channels were blocked; thus, calcium levels diminished to nearly normal levels. When normal and epileptic cells were challenged with a glutamate stimulus to increase intracellular calcium levels, restored calcium levels were quickly observed only in normal cells. In contrast, epileptic cells did not restore calcium levels even after 4 hours, and depending on the duration of the first crisis, for up to 1 year [6]. These findings evidenced that for epilepsy induction, ON mechanisms are important, but OFF mechanisms play an equally important role in this process. Other authors have seen that intracellular calcium remained elevated in neurons isolated 1 year after induction of epilepsy with pilocarpine, in comparison with normal cells in similarly aged animals [7]. These findings strongly support the notion that for epilepsy maintenance, OFF mechanisms are truly important, perhaps in a more important fashion than ON mechanisms.

In a region that has been damaged, neurons are exposed for hours to high glutamate concentrations, promoting an important inflow of calcium into the cell. This phenomenon, together with a slow removal of calcium, leads to a more prolonged period of depolarization. These abnormal changes provide epileptic cells with the ability to produce paroxystic depolarizations (PD) [8], as described for the first time by Matsumoto and Ajamone-Marsan [9]. PD are characterized by a burst of high-frequency action potentials (AP) accompanied by a sustained depolarization, which frequently is followed by hyperpolarization. Calcium participates in the activation of a phenomenon termed intrinsic burst firing, associated with synchronization of PD firing among neurons [10]. Calcium-induced glutamate exocytosis, which in turn induces the same plastic changes in efferent neurons, extends and propagates the epileptic focus.

2. LONG-TERM CELLULAR EFFECTS OF EPILEPSY

Upon an increase in the discharge of afferent connections into a neuron, the intensity of the neuron's response to these stimuli increases, i.e., potentiation of the synaptic transmission occurs. An important inflow of calcium and a calcium-induced expression of early expression genes such as c-jun, which is implicated in apoptosis-associated gene transcription; and c-fos, related with neurotrophic gene transcriptional activation. Neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin 4/5 (NT4/5) and glial cell line-derived neurotrophic factor (GDNF) also intervene in the maturation, survival, and proliferation of specific neuronal populations [1]. Such activation induces the formation of new synaptic contacts, many of which are aberrant, causing the sprouting phenomenon and imposing conditions under which excitability is exacerbated [11].

On the other hand, one consequence of glutamate receptor activation comprises an increase in intracellular calcium levels, which are finely regulated by different homeostatic cell mechanisms. Once these mechanisms are exceeded, cell death is induced through two different modalities: the acute (necrotic), or the long-term (apoptotic) death. The acute form is caused, among others, by the cell's persistent calcium entrance-mediated depolarization, causing increased sodium and chloride inflow. These ions increase water inflow into the cell, producing swelling and cell lysis [6]. In contrast, the long-term form of cell death consists of an initial increase of calcium considered non-cytotoxic since extrusion mechanisms are working. Nevertheless, as calcium extrusion mechanisms are not working properly, intracellular calcium concentration increases, allowing further liberation of this cation from intracellular stores [12]. Moreover, calcium inflow activates phospholipase A_2 [13] and nitrous oxide synthetase [14], which result with an increase in arachidonic acid and nitrous oxide. By means of cyclo- and lipoxygenase, arachidonic acid is transformed to release superoxide (O_2^-) and hydroxyl (OH) radicals. When nitrous oxide reacts with superoxide radicals, peroxynitrite ($ONOO^-$) a highly reactive molecule is generated [14]. These free radicals destroy cytoskeletal proteins, nucleic acids, and membrane lipids [15]. Peroxides, together with an increase in mitochondrial calcium permeability, alter mitochondrial function and therefore, adenosine triphosphate (ATP) generation. In addition, caspases are activated and these in turn promote the phenomenon of apoptosis. Calcium activates enzymes such as calpain, whose activation has been associated with an action upon the cytoskeleton, receptor proteins, G proteins, and calcium-dependent proteins.

3. CONTROL OF INTRACELLULAR Ca^{2+} CONCENTRATION

Cell calcium inflow is mediated by several mechanisms that together make up the named "ON component". Among these, the glutamate receptor-mediated synaptic mechanism is one of the most important since it has been extensively reported that its liberation in epileptogenesis is greatly increased. A secondary mechanism is mediated by voltage-dependent calcium channels, that is also activated during epileptic crises [1]. Internal calcium reservoirs such as endoplasmic reticulum and mitochondria intervene in the phenomenon, in a more limited manner.

On the other hand, there are specialized mechanisms to remove cytoplasmic calcium representing the "OFF component". Located in intracellular compartments important calcium uptake mechanisms are associated with the sarcoendoplasmic reticulum, the mitochondria, and the Golgi apparatus [16,17]. Also, the presence of calcium buffering proteins such as parvoalbumin, calbindin, and calmodulin (CaM) must be mentioned. However, due to their limited capacity to retain calcium, these soluble proteins participate to a greater extent in modulating calcium signals than in controlling the cation's cytoplasmic concentration. At the plasma membrane level, both the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (NCX) work against a high-concentration gradient; therefore, they are directly or indi-

rectly ATP-dependent and susceptible to ischemic injury [18]. The PMCA presents a high affinity and low capacity for removing calcium from the cell, pumping the ion against the concentration gradient; whereas the NCX is a low-affinity and high-capacity system. NCX transports sodium and calcium in opposite directions taking advantage of the sodium gradient across the membrane maintained by the Na^+/K^+ ATPase. The exchanger works in situations in which there is a large intracellular calcium accumulation that requires removal in brief periods of time, whereas the PMCA subsequently pumps the remnant calcium until the ion reaches normal values.

Alternative mechanisms of Ca^{2+}-ATPase regulation are directly coupled with specific signaling pathways according to their presence in excitable or non-excitable cells. Inherent functions of excitable cells such as neurons require the coordinated extrusion of Ca^{2+} through the PMCA and the NCX in the plasma membrane. These transporters work in parallel by means of mechanisms not fully understood.

4. MEMBRANE CALCIUM EXPORTING SYSTEMS AT THE PLASMA MEMBRANE LEVEL

4.1. Plasma Membrane Calcium ATPase (PMCA) Isoforms

PMCAs are highly conserved ATPases present in eukaryote cells that are encoded by four genes producing the basic PMCA isoforms (PMCA1–PMCA4). A large variety of isoforms, theoretically >30 PMCA variants, are generated by alternative splicing and differentially expressed according to

Table 1. Human PMCA Isoforms and Major Alternative Splice Variants

Isoform	Major Alternative Splice Variants	Tissue Distribution
PMCA1	PMCA1x/a	Brain, nervous tissue
	PMCA1x/b	Ubiquitous
	PMCA1x/c	Skeletal muscle, heart
PMCA2	PMCA2w/a	Inner ear hair cells
	PMCA2x/a	Brain (relatively rare)
	PMCA2z/a	Brain (generally more abundant than 2x/a)
	PMCA2w/b	Brain, breast (lactating mammary gland), pancreatic ß-cells
	PMCA2x/b	Brain, excitable tissue
	PMCA2z/b	Brain, excitable tissue
PMCA3	PMCA3x/a	Brain
	PMCA3z/a	Brain (cortex, thalamus, substantia nigra), pancreatic ß-cells
	PMCA3x/b	Brain
	PMCA3z/b	Brain, (cortex, thalamus, substantia nigra), pancreatic ß-cells
	PMCA3x/f	(fast) skeletal muscle, brain (rare)
PMCA4	PMCA4x/a	Brain, heart, stomach
	PMCA4z/a	Heart, pancreas (Islet of Langerhans)
	PMCA4x/b	Ubiquitous
	PMCA4z/b	Heart

Modified from Strehler *et al*., 2007.

cell type and specific function in diverse organisms (Table **1**) [19-22]. Such a high level of redundancy must represent a biological advantage for a large number of organisms, especially at the tissue level where a wide range of isoforms are present. Alternative splicing occurs at two main sites of the PMCA coding gene: site A is localized near the first intracellular loop phospholipid-sensitive region, and site C, in the CaM-binding site (Fig. **1**). In general terms, site A-edited isoforms show differences in the first intracellular loop length, whereas site C-edited isoforms exhibit differences at the C-terminal end. Regions for catalytic function, such as the ATP binding site, phosphorylation, and folding structural motifs, are highly conserved. Alternative splicing at site C is more complex due to the generation of multiple variants by inclusion/exclusion of different number of exons, which can be additionally edited through the use of internal splicing-donor sites (Fig. **2**) [19]. A controversial splicing product at site B considered aberrant, would theoretically produce a PMCA with nine transmembrane domains (k isoform) as reported in human heart, rat liver and human corneal epithelium [23-25]. According to these findings, the reorganization of the pump to a one containing eight transmembrane domains in cells expressing a k isoform [26], should be considered in further detail.

The PMCA as the ion transporter catalytic entity presents a complex and dynamic regulation. These ATPases are mainly modulated by calcium as well as by CaM, among several alternative regulators [27,28]. The alternative site-C splicing region mainly on variants "a" and "b", affects CaM binding and therefore its modulation capacity [29].

When the concentration of calcium in the vicinity of the pump corresponds to <50–100 nM, the majority of PMCAs units are inactivated and maintained in an auto-inhibitory state. In this case, the C-terminal tail makes intramolecular contacts with the 1st and 2nd cytoplasmic loops and hides the largest catalytic domain, diminishing its affinity for calcium (Fig. **1**) [16]. Therefore, the calcium-CaM domain releases the inhibition effect upon the ATPase, increasing calcium affinity and also increasing the V_{max} of the reaction. Therefore, affinity for CaM in the distinct PMCA isoforms depends on the diverse spliced forms at the C-terminal end, where the highest affinity is observed with PMCA2b (K_D <2 nM), followed by PMCA2a and -4b (K_D <5–10 nM), and finally, by PMCA4a (K_D <50 nM) [30]. The different affinities for CaM demonstrate that distinct PMCA isoforms possess different half-times of activation and inactivation constants playing specific roles during the regulation of cytoplasmic calcium concentration [31].

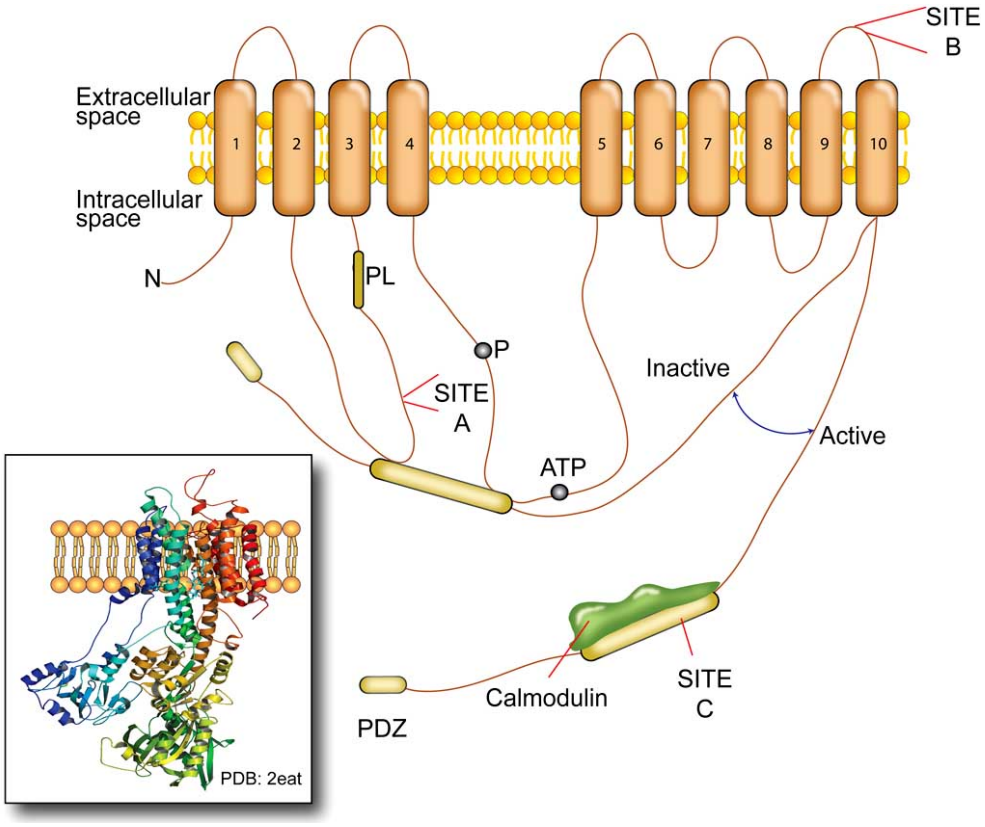

Fig. (1). Topologic representation of PMCA. This model is based on the known structure for the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA, shown in inset, PDB access number 2eat). Putative transmembrane segments (1–10), the ATP binding site, the aspartic residue in which phosphorylation (P) takes place, the binding site for phospholipids (PL) near the splicing site denominated A, and the binding site for the modulator protein calmodulin contained at the splicing site denominated C are indicated. When calcium concentration in the vicinity of the pump is <50–100 nM, PMCAs are inactivated and maintained in an auto-inhibitory state. In this state, the C-terminal tail makes intramolecular contacts with the 1st and 2nd cytoplasmic loops and hides the largest catalytic domain, diminishing its affinity for calcium. The PDZ (PSD95/DlgA/zonula occludens-1) binding domain in some "b" isoforms involved in the interaction with partner proteins is also shown.

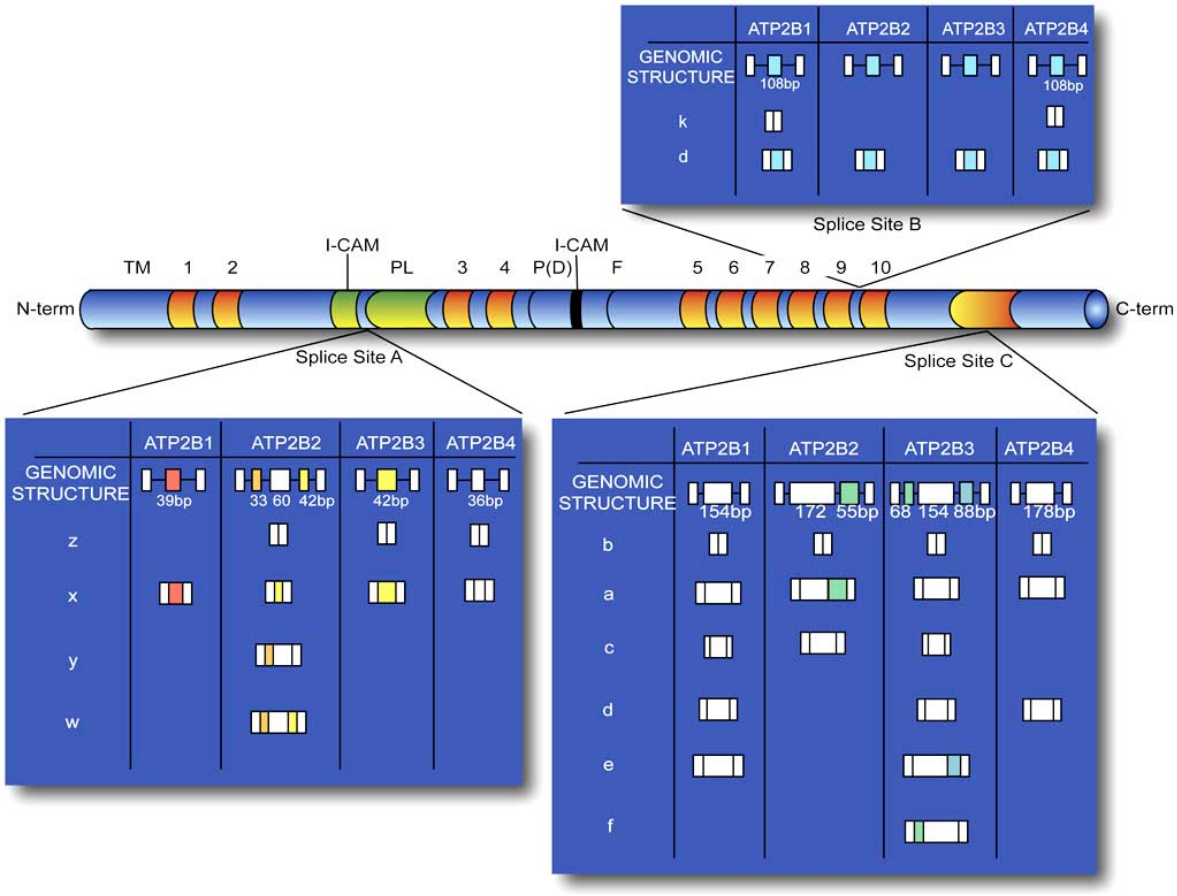

Fig. (2). Alternative splicing sites of the gene encoding for PMCA. Site A is located close to the phospholipids sensitive domain that involves a different number of exons that can be alternatively spliced. Site C located in the CaM binding domain, involves a complex splice pattern since several exons with internal donor splicing sequences can produce a diversity of PMCA isoforms with differential kinetic and distribution properties. Site B placed between transmembrane domains 9^{th} and 10^{th} is a controversial site since its physiological meaning is still not well understood. In the context of epilepsy, more research has been focused on isoforms spliced at site C, although some evidence suggests that isoforms spliced at other sites should not be excluded. Modified from [21, 22].

The different PMCA isoforms have significant differences in their capacity for managing calcium inflow of distinct intensity and duration; for instance, PMCA4b is activated by CaM at an activation rate of 46 seconds vs. 20 seconds for PMCA4a. After removing CaM, PMCA4b inactivation rate moves to nearly 20 minutes, whereas for PMCA4a this rate corresponds to <1 minute [30]. These data suggest that isoform PMCA4b is efficient for managing a slow calcium inflow, in comparison with PMCA4a, which corresponds to a competent isoform that responds to fast calcium signals [30]. In addition, since isoforms PMCA2a and PMCA3f present the highest activation rates, these are more adequate for managing a rapid calcium inflow such as that of excitable cells. PMCAs exhibit a specific memory for past activation; therefore, the inactivation constant is very slow, causing CaM to be attached to the protein for more time [32]. Thus, in cells activated by repetitive stimuli, PMCA2b is maintained pre-activated for a more prolonged period of time and therefore, responds immediately to the new calcium signal, crucial in epileptic cells.

Other PMCA modulators include several A and C protein kinases (PKA, PKC), proteases such as calpain or caspases, acid phospholipids, and dimerization or oligomerization of the enzyme [33-35]. The C-terminal region possesses abundant serine and threonine residues, the substrate for protein kinases A and C. PKA activates the pump, diminishing the Km for calcium and increasing the V_{max}. The effects of PKC are more complex and vary according to isoform and splice type. PMCA4b isoform is activated by PKC, while the PMCA4a isoform is not affected. In contrast, PMCA2a and PMCA3a are slightly inhibited [36].

The lipid environment also intervenes in the regulation of the PMCA activity, specifically through the presence of cholesterol [37-40]. Other lipids affecting PMCA regulation are acidic phospholipids, in particular, phosphatidylinositol and phosphatidylserine whose activation is partially dependent on CaM [41,42]. Interestingly, PMCAs are also affected by peptide hormones acting by means of G proteins, by steroids, and by lipid 2nd messengers such as ceramide and sphingosine [43,44].

In general, PMCA, as well as NCX function in concert with other calcium-sequestering mechanisms, such as those present in the mitochondria and the endoplasmic reticulum. Cells must be capable of precisely controlling the type, localization, and activation state of each PMCA by means of pro-

teins that interact specifically with the different PMCA isoforms [45]. As dynamic participants in the regulation of intracellular calcium, PMCA isoforms require at the long-term a fine regulation that involves changes at the transcription level, stability of mRNA, alternative splicing, and control of protein translation. At the medium-term, local availability is regulated by directioning toward the specific membrane, internalization, and recycling. This type of regulation is probably due to the intervention of partner proteins belonging to the membrane-associated guanylate kinase (MAGUK) family of proteins, which interact with the carboxy end of the 2b and 4b PMCAs [46] at specific sites such as PDZ PSD95/DlgA/zonula occludens-1 (PDZ) domains. Short-term regulation provided by CaM or by differential phosphorylation is specific for each isoform, allowing dynamic PMCA-function regulation that ranges from seconds to days [47] .

Another important factor comprises the subcellular organization of organelles involved in calcium regulation and its spatial relationship with plasma membrane microdomains. Although the manner in which these microdomains are established is not well known, it is well established that receptors, transporters, and signaling molecules are grouped into multiprotein complexes localized at strategic calcium signal sites [48].

4.2. Tissue Distribution and Local PMCA Expression

All cells express at least one PMCA variant with certain specificity for its expression in time and space. Each cell type possesses distinct splicing regulation mechanisms that determine its specific expression. PMCA1 is found in practically all cell types and has been found from the first stages of embryonic development. PMCA4 is also found in nearly all cell types, but in less abundance with respect to PMCA1 and only detected in late stages of embryonic development [49]. In general terms, PMCAs are more abundant and diverse in brain than in other tissues, particularly variants PMCA2 and PMCA3 [16,50,51].

In the brain, where highly regulated signal transduction events take place, localization of the distinct isoform variants varies greatly among its different structures and cells. For instance, PMCA1 is expressed in cerebral cortex and in the CA1 region of hippocampus [21]. PMCA2 is expressed to a greater degree in cerebellum and also in the cerebral cortex and hippocampus, while PMCA3 is abundant in superficial layers of cerebral cortex and in the cerebellar cortex. PMCA4 expression is reduced in cerebral cortex layers II and VI of pyramidal cells, as well as in cerebellar cortex granular cells. PMCA4 expression in the olfactory bulb, hippocampus and striated nucleus is very marginal.

PMCA isoforms also present a distinct distribution at the subcellular level. For example, in cerebral cortex isoforms 1 and 3 are found in the neuropile (perhaps even in the synapses) and in some dendrites. PMCA2 is abundant in distal dendrites, while PMCA4 is found as abundant in somatodendritic regions [52,53]. These differences reflect functional calcium-regulation differences in the distinct cellular compartments. The possibility exists that several isoforms could be integrated into multiprotein complexes, e.g., PMCA2 and PMCA4 that might bind to the Na^+/H^+ exchanger regulator factor and PMCA2 to the MAGUK family of proteins, that in turn bind to the NMDA receptor. PMCA4 interacts directly with the synthetase I nitrous oxide, known to decrease its activity in a dose-dependent way when calcium concentration in the microenvironment surrounding the enzyme is low [54].

Additional knowledge is also provided by studies focused on the association of specific PMCA isoforms to other proteins in caveolae related to calcium handling, since co-localization of PMCA and these structures has been suggested for different tissues [55,56]. In this sense, the association of PMCA isoforms to raft domains has been specially shown in neurons with the PMCA2 isoform [57]. However, the relevance of this type of associations in the context of epileptogenesis is still open to investigation.

4.3. Na^+/Ca^{2+} Exchanger (NCX) Isoforms

The Na^+/Ca^{2+} exchanger (NCX) is activated when intracellular calcium increases, removing one calcium ion for two or three sodium ions secondary to the Na^+/K^+ ATPase-generated Na^+-gradient formation [18], and suggested that when altered might contribute to the establishment of epilepsy [58]. The NCX1 gene in humans is localized in chromosome 2 (p23–p22), NCX2 in chromosome 19 (q13.3), and NCX3 in chromosome 14 (q24.1) (NCBI Entrez Gene database).

To date, four Na^+/Ca^{2+} exchanger genes have been cloned and functionally characterized. Isoform NCX1 was cloned in 1990 [59,60]; later, isoforms NCX2 and NCX3 were cloned in mammals. Recently, isoform NCX4 has been found in teleost, amphibian, and reptilian; and seems to be absent in birds and mammals [61,62]. Genes codifying for NCX comprise six exons (A–F); NCX1 contains all six, while NCX2 contains only two (B-C) and NCX3 contains four (A, B, C, E). NCX1 homolog genes have been identified in several species of microorganisms and have been grouped in the SLC8 (solute carrier 8) superfamily, from which the most frequently studied is NCX1 [63,64]. NCX expression levels are directly correlated to calcium extrusion in distinct cell types depending on their type of activity; for example, expression is high in cardiac cells, neurons, and kidney cells. Contrariwise, expression is relatively low in hepatic cells.

The portion corresponding to the intracellular loop oriented toward the extreme C-terminal of NCX produced by alternative splicing, vary with each one of the isoforms [65]. The 1.1–1.12 variants and the 1.41 were found from NCX1 [66]. NCX3 possesses the 3.1–3.3 variants and only the 2.1 variant is known for NCX2 (Fig. **3**).

NCX1 presents nine transmembrane domains; domains 1–5 are separated from domains 6–9 by an intracellular loop representing more than one half of the protein [67]. Two conserved regions are localized in the intracellular loop; Ca^{2+} binding domains CBD1 and 2 [68]. The transmembrane regions are responsible for sodium and calcium transport through the cellular membrane [69]. There are two other conserved regions among the several NCXs and homologs termed α1 and 2; the first one found between transmembrane regions 2 and 3, while the second is located between regions 7 and 8 [64,70]. The α1 region of the NCX1 isoform is extracellular while the α2 region is intracellular (Fig. **4**) [67].

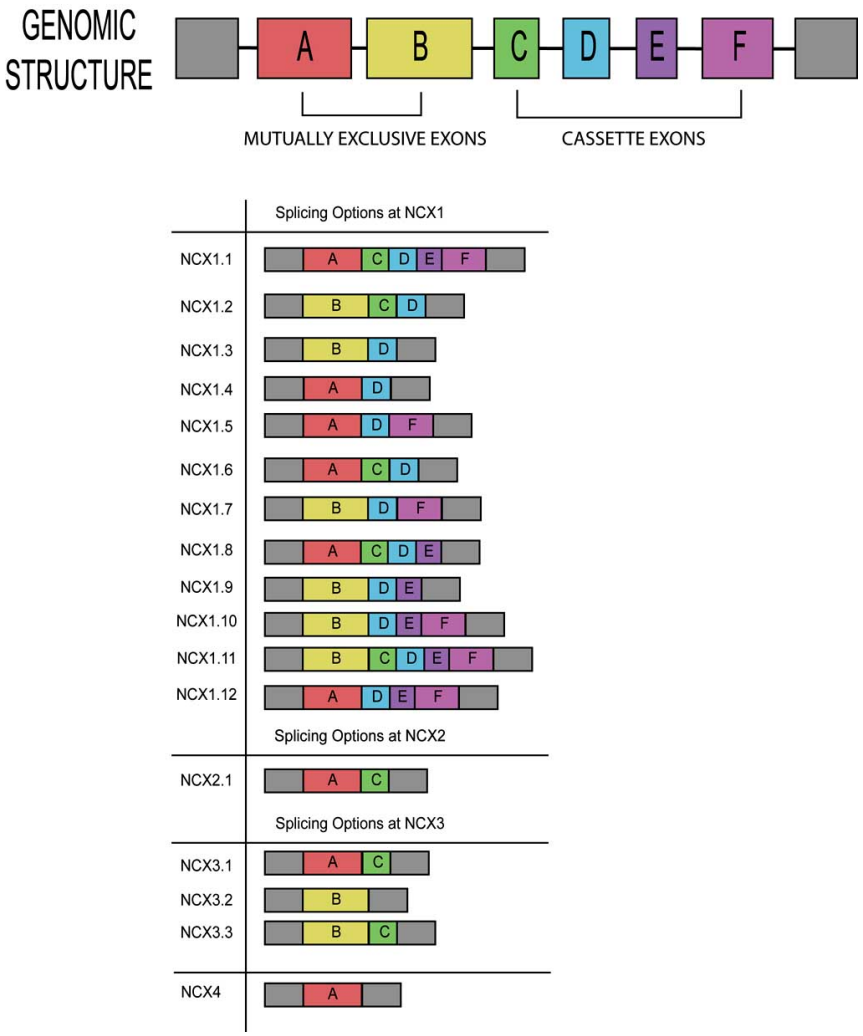

Fig. (3). Genomic structure of the NCX 1-4 isoform of the Na^+/Ca^{2+} exchanger. NCX4 is present only in teleost, amphibian, and reptilian [62]. The different alternative options are illustrated. Modified from [65].

NCX1 transports in opposite directions three sodium ions for each calcium ion moved, and depending on local ion gradients can extrude from or incorporate calcium into the cell [60]. The exchanger is coupled with the sodium gradient, on which uptake velocity as well as transport direction depends. The NCX possesses two control levels; one comprises an intracellular loop with non-proteic factors that regulate protein's activity, and the other loop interaction with other regulator proteins that also modify its activity. Sodium and calcium ions, in addition to being transported also function as NCX activity regulators; for example, the increase in intracellular sodium inactivates the exchanger (sodium-dependent inactivation, I_1). Intracellular loop removal by means of α-chymotrypsin treatment eliminates NCX inhibition mechanisms, supporting the fact that these mechanisms are indeed found in this region [60]. Other protein-regulating factors include pH, phosphatidylinositol 4,5-bisphosphate (PIP_2), ATP, phosphorylation [71]; as well as the exchanger inhibitor peptide (XIP), which completely inhibits activity when interacting with the N-terminal segment of the large intracellular loop [69]. There are a number of proteins that associate with the NCX1 in the intracellular loop that regulate exchanger transport function, such as protein kinases A (PKA) and C (PKC) [72-74]. It has been also found that phosphatases PP1 and 2A are associated with NCX1. Kinases and phosphatases are probably associated with the exchanger through another scaffolding protein, the mAKAP. Calcineurin (PP2B) is associated with the three NCXs by means of the CBD1 repeated sequence [60].

4.4. Tissue Distribution and Cell Expression of the Na^+/Ca^{2+} Exchanger

NCX1 isoform is found in the majority of cell types, while NCX2 and NCX3 isoforms are present in skeletal muscle and the nervous system (Table **2**) [75]. In the nervous system, NCX isoforms are expressed in several cerebral areas that even overlap; in particular, neocortex, cerebellar cortex, hippocampus, and hypothalamus [17]. Several isoforms are expressed in the neocortex and hippocampus, importantly in dendrites [76] and poorly expressed in axonic fibers and terminals suggesting that diverse isoforms are situated for buffering intracellular calcium in excitatory postsynaptic sites. Noteworthy, a functional interaction has been found between NCX and the glutamate receptor (mGlurR1), most probably related to calcium entry into the cell [77].

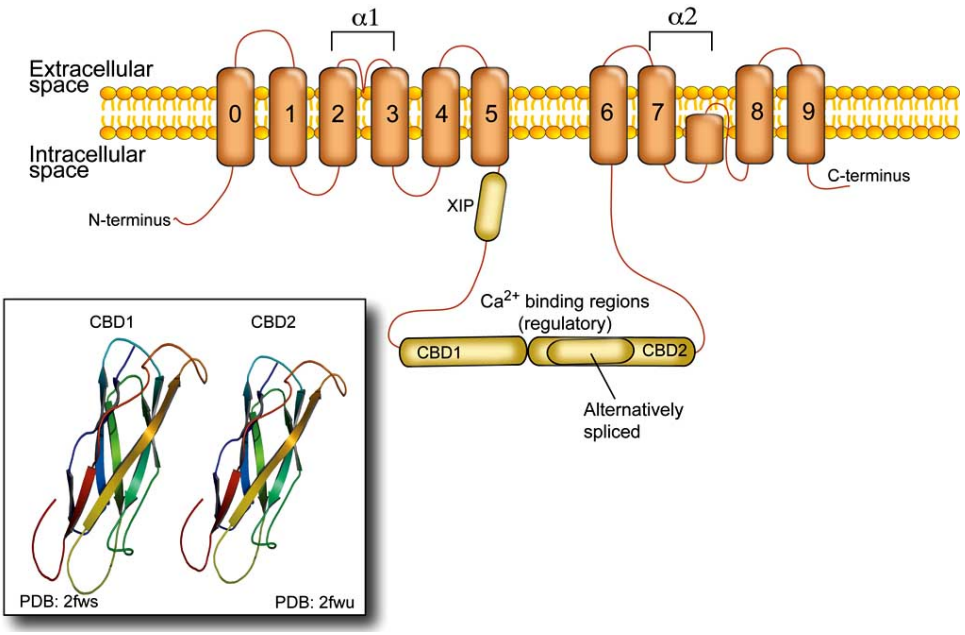

Fig. (4). Topologic representation of the Na^{+}/Ca^{2+} exchanger (NCX1). The two segments containing transmembrane domains 1–5 and 6–9 are separated by a large intracytoplasmic loop. This loop includes two domains for Ca^{2+} binding (CBD1; PDB access number 2fws, and CBD2; PDB access number 2fwu, shown in inset). There are two other conserved regions, the α1 and -2; the first is found between transmembrane regions 2 and 3, while the second is located between regions 7 and 8. These regions are considered responsible for sodium-calcium binding and transport. In the NCX1 isoform, the α1 region is extracellular, while the α2 region is intracellular. Modified from [64, 68].

Table 2. NCX Isoform and Major Alternative Splice Variants

Isoform	Alternative splice variants	Tissue distribution
NCX1	1.1	Heart
	1.2	Kidney
	1.3	Kidney
	1.4	Brain
	1.5	Brain
	1.6	Brain
	1.7	Kidney
	1.8	Brain
	1.9	Brain
	1.10	Brain
	1.11	Brain
	1.12	Brain
NCX2	2.1	Brain and skeletal muscle
NCX3	3.1-3	Brain and skeletal muscle

5. ROLE OF PMCA AND NCX IN EPILEPSY

Within the context of epilepsy, little is known concerning the role that distinct PMCA isoforms play in the control of epileptic crises and their consequences. Mapping studies conducted with *in situ* hybridization techniques in hippocampus of intraperitoneal (i.p.) kainic acid (KA)-treated rats, exhibited a differential expression of PMCA isoforms 1–3 [78]. In the case of isoform 1, there are no appreciable changes in mRNA levels in CA1 and 3 pyramidal cells up to 4 hours after injection with KA. At 12 hours, there is a significant decrease (47%) that is maintained up to 72 hours in CA3. In the dentate gyrus, no significant changes were observed for PMCA1. In the case of PMCA2, a maximum decrease is achieved at 12 hours post-injection, reaching levels ca 40% less than controls in CA3 and 1 and a maximum decrease of PMCA2 is achieved at 4 hours, reaching 34% less than controls. PMCA3 does not show significant changes in the entire hippocampus during the observation period. Complementary western blot analysis demonstrates that total PMCA protein levels were higher than controls only at 4 hours after treatment with KA [78]. Furthermore, in the inner molecular layer of the dentate gyrus of KA-treated rats, a higher expression of total PMCA was detected by immunocytochemical techniques at 3 weeks and 2.5 months after the injection of KA [79].

Based on this type of experimentation, it is recognized that clearance of calcium mediated by the PMCA has a relevant role in different neuronal processes. Elegant experiments performed with hippocampal cells expressing different PMCA isoforms fused to the enhanced green fluorescent protein have consistently shown that glutamate through NMDA receptors induce a Ca^{2+} inflow. Moreover, through the selective inhibition of processes such as the activity of SERCA, mitochondrial Ca^{2+} uptake, or NCX activity, it has

been shown that PMCA is the main protein mediating the extrusion of cytoplasmic calcium [80]. These studies have also shown that PMCA isoforms 4b, 2wb, and 2xb are internalized in hippocampal cells after glutamate exposure even at non-toxic concentrations. The observed internalization appears not to be due to damage of the plasma membrane, but instead mediated by calpain through an unknown mechanism [80].

New data from our laboratory employing single cells recorded with a whole-cell configuration and real-time PCR to measure different PMCA mRNAs, show that the expression of the housekeeping isoform PMCA1 importantly increases in cells localized in the hippocampus of chronic pilocarpine-induced epileptic rats [Bravo-Martínez *et al*., submitted]. In contrast, PMCA3 transcripts show a significant decrease in the epileptic condition [Bravo-Martínez *et al*., submitted]. These results might be interpreted in the context that different PMCA isoforms displaying a wide range of affinity constants for calcium and CaM might be specifically adjusting to the new condition. It is interesting to note that PMCA1 and PMCA4 as housekeeping isoforms have been considered slow-acting proteins, whereas PMCA2 and PMCA3 isoforms have been considered as fast-acting proteins in the fine tuning of cytoplasmic calcium concentrations [81].

On the other hand, it is noteworthy that nowadays less knowledge is available concerning the role of NCX during epileptogenesis. In the hippocampus of KA-epilepticized rats, it was demonstrated by means of immunocytochemistry techniques that the NCX1 isoform diminished in the internal molecular layer of the hippocampus as well as in layer III of the entorhinal cortex; whereas the NCX2 isoform increased mainly in astrocytes. Concerning the NCX3 isoform, it has been shown to diminish in mossy fibers, probably promoting the phenomenon of sprouting, since an increase in intracellular calcium is required for this process to occur [79].

So far, although the specific participation of Ca^{2+} extruding systems in epileptogenesis such as the one given by the PMCA is still unknown, useful information can be obtained from related studies. For instance, it has been shown that cultured neuroblastome cells exposed to depolarizing KCl concentrations induce the expression of isoform 2x, while during basal conditions it is not found [82]. Interestingly, the effect is observed for several generations and demonstrated that a calcium transient is a necessary condition to express the 2x isoform in addition to the 2w found in non-stimulated cells [82]. Complementary information is added by studies conducted with cultured hippocampal neurons during maturation, where an important upregulation at the level of tran-

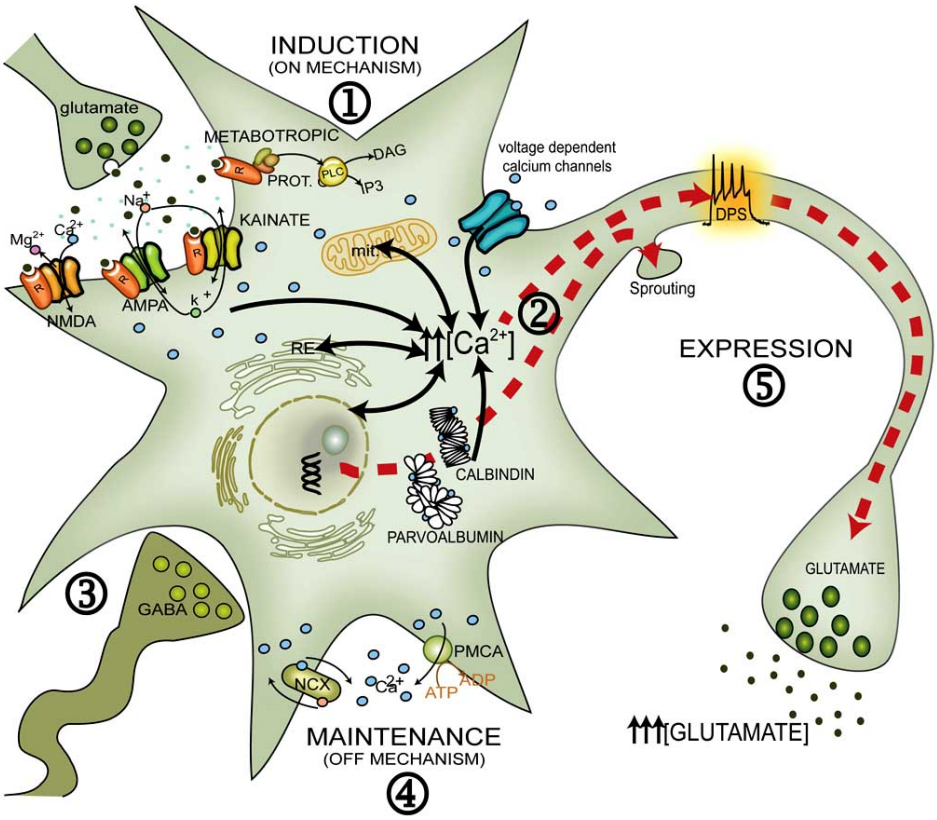

Fig. (5). Mechanisms of epileptogenesis and maintenance of epilepsy. Events related with epilepsy induction (1) involve stimulation mediated by excitatory neurotransmitters (adrenergic, cholinergic, glutamatergic) and by iterative electric stimulation, both resulting in a significant Ca^{2+} inflow (2). This entrance of Ca^{2+} is carried out by ON mechanisms located in the plasma membrane (glutamate receptors and voltage-dependent calcium channels). For epilepsy to be accomplished, the GABAergic inhibitory systems (3) must show an altered function producing an imbalance in the homeostasis between excitatory and inhibitory neurotransmitters. In the maintenance stage of epilepsy (4), calcium levels reach a higher new threshold near a normal concentration due to inadequate function of OFF mechanisms, basically the PMCA and the Na^+/Ca^{2+}exchanger (NCX). A sustained elevation of intracellular Ca^{2+} levels is an essential condition for the cells to fire paroxystic depolarizations and to synchronize with other cells in order to express clinical and electroencephalographic manifestations (5).

scripts and proteins mainly PMCA2 and the NCX2, has been described [83]. During neuronal maturation it is assumed that nervous cells require to improve their Ca^{2+} buffering capacity, and that changes might occur concomitantly with changes within intracellular Ca^{2+} buffering systems [83].

6. CONCLUDING REMARKS

Up to date, accumulated knowledge points to the fact that the ultimate control of calcium homeostasis in the neuron is carried out at the level of the plasma membrane by the Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger (Fig. **5**). In the case of the epileptic cell, a different cytoplasmic calcium concentration threshold than the one observed in normal cells, is maintained within manageable levels. This higher intracellular calcium concentration induces the cell to discharge paroxystic depolarizations and to synchronize with the remainder of epileptic cells. In parallel, a new equilibrium is reached in challenged cells where the expression of specific PMCA isoforms and other proteins mobilizing calcium lead the cell to overcome the new physiological demand. The temporal screening for the expression of the different isoforms providing the adequate kinetic properties aimed to restore as much as possible the "normal" cytoplasmic calcium levels, is the focus of future investigation. Likewise, the search of alternative regulating mechanisms involved at the transcriptional and/or translational levels of these isoforms will permit a better understanding of the origin and possible control of epileptogenesis.

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