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PLASMA MEMBRANE CALCIUM ATPASES AS CRITICAL REGULATORS OF CALCIUM HOMEOSTASIS DURING NEURONAL CELL FUNCTION

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1. ABSTRACT

The plasma membrane calcium ATPases (PMCAs) are ubiquitously expressed proteins that couple the extrusion of calcium across the plasma membrane with the hydrolysis of ATP. In mammals, four separate genes encode distinct PMCA isoforms. Complex patterns of alternative RNA splicing generate additional isoform variability. Functionally, the PMCAs were originally assigned the role of maintaining basal levels of intracellular calcium. Recent evidence, however, is expanding the role of the PMCAs as important participants in dynamic Ca^{2+} regulation and as crucial players of Ca^{2+} export during normal and pathological conditions. This review highlights recent advances made on the biology of the PMCAs within the context of neuronal development, cellular responses to external stimuli and cell survival. Particular emphasis is placed on the role of the PMCAs in vestibular and auditory functions, localized calcium signaling in photoreceptor synaptic terminals and calcium-mediated cell death.

2. INTRODUCTION

The calcium ion (Ca^{2+}) acts as a ubiquitous second messenger signal in the control of eukaryotic cell function. Changes in the intracellular Ca^{2+} concentration are therefore under tight spatial and temporal control. Ca^{2+} entering the cell from the extracellular space increases the total calcium concentration present in that cell. Over time, unchecked Ca^{2+} entry may lead to Ca^{2+} overload, eventually saturating the intracellular buffering and organellar storage systems. The maintenance of Ca^{2+} homeostasis therefore requires the presence of Ca^{2+} extrusion systems in the plasma membrane that compensate for the Ca^{2+} influx from the extracellular milieu. The two plasmalemmal systems primarily responsible for Ca^{2+} extrusion are the plasma membrane Ca^{2+} ATPase (PMCA) and the Na^{+}/ Ca^{2+} exchanger (1-3).

The plasma membrane calcium ATPase (PMCA) was first isolated from erythrocyte membranes over thirty years ago (4) and was one of the first enzymes shown to be regulated by the calcium sensor protein calmodulin (1, 5). Subsequent work has determined that the PMCAs are generated from a gene family consisting of four distinct members, designated ATP2B1, ATP2B2, ATP2B3, and ATP2B4 in the human genome database (GDB) nomenclature. The four major isoforms encoded by these genes (PMCAs 1-4) are expressed in a tissue specific manner. PMCA isoforms 1 and 4 are considered house keeping forms because of their almost ubiquitous expression. In rats and humans, PMCA isoform 1 has been detected in all tissues examined to date (6-9). In humans, PMCA isoform 4 has also been found in all tissues examined (9-11) whereas in rats, this isoform is less abundant and appears to be absent in liver (12). PMCA isoform 2 is expressed primarily in brain and heart, and PMCA isoform 3 is essentially confined to brain and skeletal muscle (6-10, 13). Further isoform variability is achieved by mRNA splicing which occurs at two locations, splice sites A and C (figure 1) (14). The functional significance for alternative splicing at site A has not yet

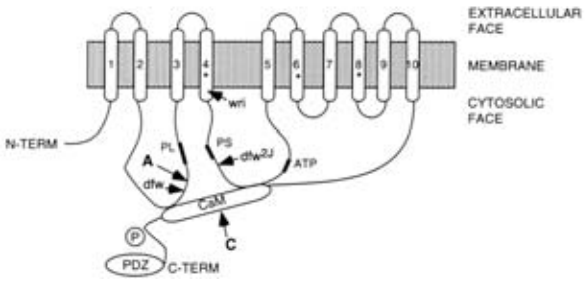

Figure 1. Topological model of the plasma membrane calcium ATPase. Note that the bulk of the protein mass is on the cytosolic side of the membrane. The transmembrane segments are clustered in two blocks (numbered 1 to 4 and 5 to 10). The PMCA is shown in the autoinhibited state in which the calmodulin-binding domain (CaM) is bound to two intramolecular sites on the first and second hydrophilic loops. The catalytic phosphorylation site (PS) and a stretch of the ATP-binding site (ATP) are indicated. A site of regulatory phosphorylation by cAMP-dependent protein kinase and/or protein kinase C in the C-terminal domain is marked by a circled P, and the region involved in phospholipid-sensitivity is labeled PL. Asterisks in transmembrane segments 4, 6 and 8 indicate the position of important amino acid ligands for Ca^{2+}. Arrows labeled A and C point to the regions where isoform diversity is created by alternative RNA splicing at sites A and C, respectively. PDZ indicates that the extreme C-terminus of some PMCA splice variants can bind to the PDZ domain found in several proteins involved in clustering and anchoring of membrane receptors and transporters. The positions where the "deafwaddler" (dfw and dfw^{2J}) and "wriggle mouse Sagami" (wri) mutations alter the pump sequence are also indicated. Note that the dfw^{2J} mutation leads to a reading frame shift and truncation of the protein within the second cytosolic loop.

been elucidated although it has been hypothesized that differential regulation by phospholipids may be involved (14, 15). Splicing at site A affects a region of the protein previously shown to be involved in lipid regulation of the pump (16-18). In contrast to the sparse information on the significance of splice site A variants, splicing events at site C have been much better characterized with respect to their functional consequences. Splicing at site C has been shown to alter the PMCA's ability to bind calmodulin and, importantly, to affect the apparent Ca^{2+} affinity (i.e., the $K_{1/2(Ca2+)}$) of the final protein product (19-21).

The PMCAs range in molecular weight from 125-140 kDa, and are members of the P-type class of ion-motive ATPases (22, 23). They contain ten predicted transmembrane domains, and the C and N-termini are located intracellularly (figure 1) (14, 23). The two major intracellular loops, located between transmembrane domains 2 and 3 and between transmembrane domains 4 and 5, comprise the transduction domain and the catalytic region of the PMCA, respectively (figure 1). The extended C-terminal tail of the pump is the main regulatory region. It contains the calmodulin-binding domain, sites for phosphorylation by protein kinases A and C, and sequences for additional protein interactions that may determine the membrane localization, targeting, and cross-talk with other signaling molecules of the pump (24-27). The calmodulin-binding sequence is thought to act as autoinhibitory domain, effectively keeping the pump in an inactive state in the absence of calcium-calmodulin (i.e., in the presence of low intracellular free Ca^{2+}) (24, 28). During times of low intracellular calcium, intramolecular interactions of the C-terminal autoinhibitory tail with two sites located within the first and second cytosolic loop prevent high-affinity Ca^{2+} binding and transport (figure 1) (3, 24, 28). The functioning of the PMCA is, therefore, regulated itself by $[Ca^{2+}]$. Although the precise mechanism of Ca^{2+} transport is not yet known, site-directed mutagenesis studies have shown that conserved amino acid residues within transmembrane domains 4, 6 and 8 are essential for calcium transport by the PMCA (figure 1) (29, 30).

Due to their high affinity for Ca^{2+} ($K_{1/2(Ca2+)}$) ≈ 0.2-0.5 μM), the PMCAs have been long believed to play a major role only in maintaining the basal level of $[Ca^{2+}]_i$ (23). Recent evidence, however, suggests a more active role for the PMCAs in returning $[Ca^{2+}]_i$ to basal levels after cellular stimulation (31-34). A more dynamic involvement of the PMCA in shaping intracellular Ca^{2+} signals is also suggested by the multitude of isoforms and splice variants showing different regulatory and transport properties. In addition, recent work has demonstrated that PMCA isoforms are asymmetrically distributed in polarized cells where they may be concentrated in specific membrane domains (e.g., in dendritic spines of cerebellar Purkinje cells) (35-37). The basolateral distribution of the PMCA (mostly PMCA isoform 1) in kidney epithelial cells (38) or its enrichment in the dendrites of cerebellar Purkinje cells (mostly PMCA isoform 2) (37) suggests that specific PMCA isoforms are actively involved in the spatial and temporal regulation of Ca^{2+} transport and signaling. Despite these recent advances in assigning the PMCAs a more active role in calcium homeostasis, their importance for the proper development and functioning of neuronal systems, for intracellular responses to external stimuli and for the survival of cells during pathological events has only recently begun to be elucidated. In this review, recent progress leading to a better understanding of the role of the PMCAs in the aforementioned processes will therefore be summarized and critically evaluated. For a comprehensive overview of the discovery and biochemical characterization of the PMCA as well as of the molecular and functional characterization of its many isoforms and splice variants, the reader is referred to reviews by Schatzmann (39), Carafoli (23, 24), Strehler (14), Monteith and Roufogalis (32), Penniston and Enyedi (33), and Guerini (40) to name just a few.

3. ROLE OF THE PLASMA MEMBRANE CALCIUM ATPASE IN VESTIBULAR AND AUDITORY DEVELOPMENT AND FUNCTION

Interest in the role of the PMCA in vestibular and auditory functions stems from recent studies that revealed a high concentration of PMCA in vertebrate

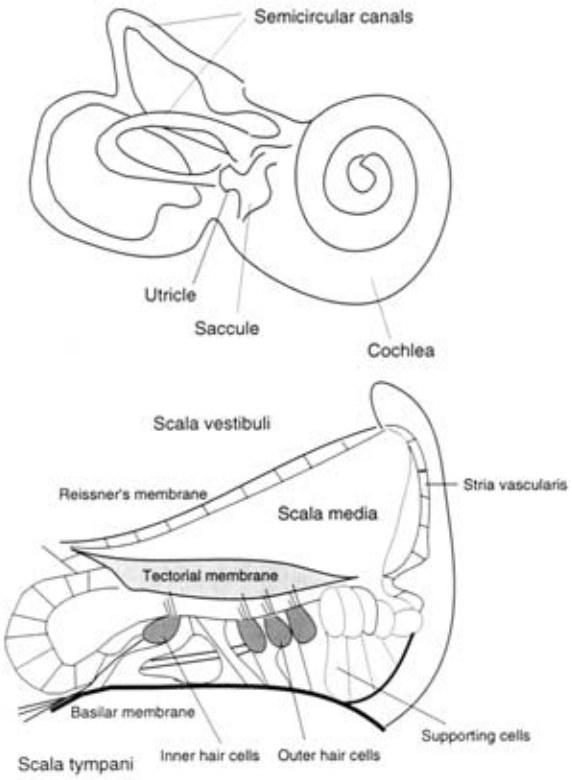

Figure 2. Schematic drawing of the anatomy of the inner ear and of a cross-section through the organ of Corti in the cochlea. The main inner ear structures are indicated on top, and a cross-section through a part of the cochlea emphasizing the organ of Corti is shown below. The cochlear inner and outer sensory hair cells are shaded, their apical stereocilia (hair bundles) are shown in contact with the tectorial membrane.

sensory hair cell bundles (41, 42) as well as distinct hearing and balance impairments in animal models due to the lack or malfunction of the PMCA (43-45). Before discussing these recent and exciting findings (see below), a brief overview of the main anatomical features of the vestibular and auditory system may be appropriately given here. The vestibular and auditory systems are located within the inner ear (46). The vestibular system is composed of the semicircular canals, utricle and saccule (figure 2). These structures contain similar sensory regions composed of supporting cells and sensory hair cells. Changes in angular acceleration are detected by movement of endolymph (a viscous fluid of specific ion composition) within the semicircular ducts, inducing movement of the stereocilia of hair cells eventually resulting in an action potential. Within the utricle and saccule, sensory regions containing hair cells are known as maculae. Maculae detect changes in linear acceleration and head position with respect to gravity. The hair bundles of the sensory cells in the maculae are attached to a gelatinous otolithic membrane which supports small crystalline bodies composed of calcium carbonate and protein, known as otoconia. Movement of the endolymph causes movement of otoconia which in turn induces deflections of the stereocilia of hair cells resulting in a membrane potential.

The auditory system is composed primarily of the vestibule and the cochlea (46). The cochlea forms the cochlear duct that can be further subdivided into the fluid-filled compartments scala vestibuli, scala media and scala tympani (figure 2). Sensory regions for hearing reside within the organ of Corti (see (47) for a brief overview). Within the organ of Corti, sensory cells are composed of outer and inner hair cells with supporting cells (figure 2). The stereocilia of the hair cells are embedded within the tectorial membrane. When the bones of the middle ear vibrate with incoming sound, they apply force that is translated into pressure waves within the perilymph of the scala vestibuli. Pressure waves force the vestibular membrane, also known as Reissner's membrane, to bulge downward into the scala media causing vibrations within the endolymph. These vibrations are transmitted to the basilar membrane, eventually causing shearing forces between hair cells and the stationary tectorial membrane. These shearing forces cause movement of the hair cell stereocilia which convert the mechanical force into a change in membrane potential.

3.1. Data from a PMCA2 knockout mouse

Mechanoelectrical transduction channels of hair cells have been shown to be selectively permeable to Ca^{2+} (48). Ca^{2+} entering via channels is important to many of the hair bundle functions such as hair cell adaptation (49). The ionic environment of endolymph is incompatible with Na^+-dependent Ca^{2+} extrusion by the Na^+/Ca^{2+} exchanger, leaving the PMCA as the main mechanism for Ca^{2+} export from the hair cell bundles (50). Accordingly, the PMCA has been shown to be abundant in hair cells as determined by immunohistochemistry using PMCA-specific antibodies (41, 42). Furthermore, elegant immunochemical and electrophysiological studies on frog saccular hair cells have shown that the extremely high concentration of PMCAs in the hair bundle membrane (estimated at about 2000 pump molecules per μm^2) is necessary and sufficient to effectively handle large Ca^{2+} fluxes across the hair bundle plasma membrane (50). Using PCR and *in situ* hybridization methods, Furuta *et al.* (51) have recently begun to address the question of which PMCA isoforms and splice variants are expressed in the developing and adult rat auditory system. These studies have shown that remarkable changes occur in the expression pattern of different isoforms in the developing cochlea. For example, PMCA2 is first detected around embryonic day 12 to 14 in the cochleo-vestibular ganglion, gradually increases in the spiral ganglion and is high in Reissner's membrane and stria vascularis at birth. Strong expression is also seen at birth in the outer hair cells. Interestingly, after the animals are about one week old, expression of PMCA2 decreases rapidly in all cochlear structures except the outer hair cells and spiral ganglion neurons. PMCA2 expression remains very high in these cell types into adulthood, and is moderate in Reissner's membrane (51). By contrast, PMCA1 - while more uniformly expressed than PMCA2 throughout most of embryonic development - displays a transitory rise in expression in the cochlear tissues during

early postnatal development and remains the major isoform in the inner (but not the outer) hair cells of the adult organ of Corti (51). Taken together, the above studies provide evidence for a major role of specific PMCA isoforms in cochlear function, however, until recently their contribution to calcium homeostasis within the stereocilia and their importance for the physiology of hearing and balance were not known.

Enter the landmark study by Kozel *et al.* (44) who recently reported on the phenotype of mice null for any functional PMCA2 ($ATP2B2^{-/-}$ mice). This study - the first to report the "knockout" of any PMCA - provided evidence that the contribution of the PMCA (at least of PMCA2) transcends regulation of basal resting levels of intracellular Ca^{2+}. Although PMCA2 null mice were viable, they displayed a range of abnormalities. These mice grew slower than both heterozygous and wild-type mice. In addition, they exhibited an unsteady gait and severe difficulties maintaining balance (44). Histological analysis of the cerebellum and inner ear of the knockout mice revealed major anatomical deformities. A detailed analysis of the cerebellum seemed of particular interest given the previous reports that PMCA2 is the major PMCA isoform expressed in that tissue (10, 37, 52). Within the cerebellum, null mutants had increases in the numbers of Purkinje neurons and a decreased thickness of the molecular layer (44). The observed increase in the number of Purkinje neurons is clearly of interest although it is currently unclear if this reflects a compensatory upregulation of cell proliferation or is due to a defect in selective neuronal death during cerebellar development. Within the vestibular system, PMCA2 null mutants had no anatomical alterations within any of the vestibular structures. Remarkably, however, otoconia were completely absent within the macula of both the utricle and saccule of null mice (44). The balance problems within the null mice could therefore be attributed to the loss of otoconia, which are extremely important for detection of both linear acceleration and head position with respect to gravity.

Major histopathological alterations were detected within the organ of Corti in the PMCA2 knockout mice (44). The organ of Corti ranged in abnormalities from slight alterations within the tunnel of Corti with hair, pillar and other cells still present to no tunnel of Corti and the complete absence of hair, pillar or other cells. As a result of abnormalities within the organ of Corti, all of the null mice tested, utilizing auditory brainstem responses, were completely deaf (44). Interestingly, mice heterozygous for the PMCA mutation ($ATP2B2^{+/-}$) were clearly hearing-impaired as their threshold for auditory brainstem responses was elevated to 70-80 db compared to 30-45 db for wildtype animals. In agreement with the physiological findings, histopathological abnormalities of varying degrees were found in the cochlear ducts of these heterozygous mice. By contrast, the heterozygous mice were not different from the wildtype littermates in their gross phenotype, i.e. they did not show the severe ataxia observed in the homozygous mutants (44). These data so far provide the most definitive support for the notion that different PMCA isoforms perform specific and specialized functions in different cells. Apparently, the remaining PMCA isoforms are unable to compensate for the lack of PMCA2 expression in the cochlea of the knockout mice, and even a reduction of PMCA2 expression (as in the heterozygous animals) results in a significant impairment of auditory function.

3.2. Recent insights from studies of the "deafwaddler" mouse and the "wriggle mouse Sagami"

A spontaneous mutation that arose within a C3H/HeJ mouse colony produces mice that are deaf, waddle while walking and bob their heads (43). The genetic locus of this "deafwaddler" (*dfw*) mutation was mapped to a region of chromosome 6 corresponding to the locus of the ATP2B2 gene for PMCA isoform 2 (43, 53, 54). The *dfw* mutation was determined to be a G to A transition in an exon of the ATP2B2 gene, resulting in a glycine to serine change at amino acid position 283 of the PMCA2 isoform. Gly-283 is located in the first cytosolic loop between transmembrane domains 2 and 3 (figure 1) and is embedded in a sequence highly conserved among all PMCA isoforms and even among many other P-type ion-transporting ATPases. The effects of altering this glycine residue have previously been studied in a H^+ ATPase from *Saccaromyces pombe* and in the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) from rabbit skeletal muscle (55, 56). In *S. pombe*, the mutation occurred spontaneously and resulted in a substitution of the glycine by an aspartate residue. Even though a glycine to aspartate change appears more drastic than the glycine to serine change present in the PMCA2 of *dfw* mice, the *S. pombe* H^+ pump still showed about 50% of its wildtype ATPase activity and displayed a reduced sensitivity to vanadate inhibition (55). In the SERCA, site-directed mutagenesis was used to alter the corresponding glycine to valine, arginine or glutamate. All of these changes are more drastic than the observed glycine to serine change in the *dfw* PMCA2, and all three SERCA mutants were unable to pump Ca^{2+} although they retained the ability to form the acyl-phosphate intermediate (E1-P state) from ATP (56). These data strongly suggest that the glycine in question is important for the conformational changes that accompany the E1 to E2 transition during the ion transport cycle. Because this glycine is not directly involved in Ca^{2+} binding or phosphorylation (as judged from the results with the SERCA mutants), it must be involved in the coupling of the conformational changes caused by the phosphorylation step to those that occur at the Ca^{2+} binding sites (presumably close to and within the transmembrane domains) leading to the movement of Ca^{2+} from the inside to the outside. Although no biochemical data are yet available on the functional consequences of the *dfw* mutant protein, it is fair to assume that the glycine to serine change also impairs the conformational coupling and thus the ability of PMCA2 to transport Ca^{2+} out of the cell. Notably, and in contrast to the PMCA2 knockout mice discussed above, homozygous *dfw* mice contain wildtype levels of PMCA2 mRNA in their auditory and vestibular tissues, and the mutant protein is expressed at normal levels and properly localized in the cochlear hair cells (43). Thus, the deafness and ataxic phenotype of the *dfw* mice is clearly due to a functional deficiency of the PMCA2 pump and not a consequence of its cellular mislocalization. It is worth

noting, however, that the *dfw* phenotype is not as severe as that of the PMCA2 null mice. For example, cochlear hair cells appear anatomically normal at birth, and distinct anatomical and physiological abnormalities of the vestibular and auditory system become manifest only as the animals grow older (43).

A second, more severe spontaneous deafwaddler mutation (*dfw^{2J}*) has also been recently characterized (43, 57). Mice homozygous for this mutation are deaf, show unbalanced movement and display slight body tremor (57). Analysis of the defect in *dfw^{2J}* mice revealed a two base pair deletion within a protein coding exon of the PMCA2 gene. This deletion leads to a change in the reading frame after amino acid 456 and results in a premature stop codon that truncates the protein after amino acid 471. The change in reading frame occurs just three residues downstream of the invariant essential aspartate residue in the major catalytic loop of the pump, and the truncated protein lacks essential domains such as the ATP binding site and all downstream transmembrane and Ca^{2+} binding regions (figure 1). Accordingly, the mutant protein is expected to be completely nonfunctional. In fact, mRNA for PMCA2 was virtually undetectable in the *dfw^{2J}* mice and immunohistochemical analysis of the inner ear revealed a dramatic decrease in the expression of PMCA within the organ of Corti and the saccule and the ampullas of these mice (43). Thus, *dfw^{2J}* homozygotes may be considered functional null mutants, a notion supported by their phenotype which is strikingly similar to that of the PMCA2 knockout mice described by Kozel et al. (44). Both the knockout and the *dfw^{2J}* mice - although viable and apparently with a normal lifespan - are unable to breed (44, 57), and their ataxic, vestibular and auditory phenotype is more severe that that of the *dfw* mice.

The most recent confirmation of the importance of PMCA2 for auditory and balancing functions stems from a molecular analysis of the genetic defect leading to yet a different spontaneous mouse mutant, called "wriggle mouse Sagami" (45). Homozygous *wri* mice suffer from a neurological disorder characterized by abnormal movements and progressive hearing loss (58). In these mice, a single G to A transition changes the codon for amino acid 412 of the PMCA2 gene from GAG to AAG, leading to the substitution of a glutamate by a lysine residue (45). Residue 412 is located in the fourth transmembrane segment of the pump (figure 1) and appears to be a crucial Ca^{2+} ligand during ion transport. When the corresponding residue (Glu-423) in PMCA4 is mutated to Ala, Asp, Asn or Gln, the resulting protein is essentially unable to pump Ca^{2+} when expressed in COS cells (29). However, formation of the phosphoenzyme intermediate from phosphate (but not from ATP) is still possible, suggesting that the mutant pumps are stabilized in the E2 conformation (29). Although a detailed study of the expression of the mutant PMCA2 in *wri* homozygous mice has not yet been reported, immunohistochemical data presented by Takahashi and Kitamura (45) indicate that the level of expression may be reduced in these animals. The *wri* mice appear normal at birth and no anatomical abnormalities are initially detected in the vestibular or auditory system. Neurological symptoms first appear at about 10 days of age (58) and progress rapidly. Hearing loss is apparent shortly after birth and severe degeneration of the cochlea and saccule is observed within three months (45). Heterozygous *wri*/+ mice show initially mild hearing impairment which progresses to severe hearing loss after one month. Because the cochlear hair cells and their stereocilia appear anatomically normal in young *wri* homozygotes and in *wri*/+ heterozgotes, the auditory deficits of these animals can not be ascribed to an absence of the appropriate anatomical structures but rather reflect the selective loss of PMCA2 function.

4. ROLE OF THE PLASMA MEMBRANE CALCIUM ATPASE IN INTRACELLULAR RESPONSES TO EXTERNAL STIMULI

Many higher processes that occur within the nervous system are a result of an external stimulus applied to a distinct subset of neurons. This stimulus induces a cascade of intracellular events, many of which involve the use of second messengers. Ca^{2+} is arguably one of the most commonly utilized second messengers. Therefore, the proper regulation of intracellular Ca^{2+} is crucial to the temporal and spatial propagation of intracellular responses to extracellular stimuli. Recent studies have contributed to a better appreciation of the role of the PMCAs in the long-term and dynamic regulation of these stimulus-induced Ca^{2+} signals.

4.1. Neuronal differentiation

Pheochromocytoma cell (PC) lines differentiate in response to nerve growth factor (NGF). The intracellular responses of PC lines to NGF are well characterized, starting with high affinity NGF receptor (TrkA) activation and leading to downstream signaling cascades that involve both the MAP kinase and the phospholipase C/inositol trisphosphate/Ca^{2+} signaling pathways (59). The most obvious and easily observed downstream event is a morphological response, i.e. the extension of neurites. At the molecular level NGF induces the phosphorylation of a number of intracellular proteins and the expression of immediate early genes. Although rapid as well as persistent changes in intracellular Ca^{2+} are involved in many of the NGF-induced processes, the role of the PMCAs in shaping these NGF-dependent physiological responses is not clear. Utilizing antisense technology, Vanaman and colleagues inhibited the production of PMCA isoform 1 within the PC6 cell line (60). Stably transfected cells expressed no PMCA1 as determined by Western blotting and showed a decrease by about 37% in total PMCA protein. The remaining 67% of PMCA were likely due to PMCA4 (which is a major isoform in many cell lines) and PMCA2, both of which were detected in the PC cell line (60). Although the PMCA1-deficient cells appeared grossly normal, they were significantly slower in returning bradykinin induced Ca^{2+} transients back to resting levels and did not attach to substrates as well as controls. PMCA1-depleted PC6 cells were also deficient in their ability to extend neurites and in some cases no neurites were formed after 6 days of NGF treatment (60). This

reduction (or loss) of NGF induced neurite outgrowth can not simply be explained by altered initial responses to NGF as PMCA1-depleted PC6 cells still responded to NGF in a similar manner as wild type cells. Specifically, both cell types phosphorylated erk 1 and erk 2 and induced the expression of the immediate-early gene NGFI-A, suggesting that NGF receptor signaling pathways were equally functional in the control and PMCA1-deficient PC6 cells (60). The loss of neurite extension in the PMCA1-depleted cells may be due to their inability to properly buffer intracellular calcium thereby affecting the affinity and/or expression levels of integrins. Preliminary studies indicated that PMCA1-depleted cells expressed reduced levels of alpha 1 integrin which is required for NGF induced neurite extension (60). However, the resting level of $[Ca^{2+}]_i$ was unaltered in the PMCA1-deficient cells compared to the wild-type controls, suggesting that the remaining PMCAs as well as the SERCA pumps were sufficient to handle the Ca^{2+} loads. The data underline once again the importance of specific PMCA isoforms not just for the maintenance of basal $[Ca^{2+}]_i$ but for the dynamic handling of Ca^{2+} transients. Thus, the slower kinetics of returning a Ca^{2+} signal to basal values in the PMCA1-depleted cells may be the crucial determinant of the observed change in phenotype of these cells.

Changes in PMCA expression have also been shown to be involved in differentiation in other cell systems, although the causal relationship between altered PMCA activity and differentiation is not yet clear. Stable overexpression of the human PMCA4b in rat L6 myoblasts resulted in a significant acceleration of cell fusion into myotubes (from about 10-12 days in control myoblasts to 6-8 days in PMCA4b-overexpressing cells) and a concomitant faster increase in creatine kinase activity (61). Fusion into multinucleate myotubes and increased creatine kinase activity are hallmarks of myogenic differentiation. In contrast to the PMCA1-deficient PC6 cells which showed no change in $[Ca^{2+}]_i$, the hPMCA4b-overexpressing L6 cells displayed a significant (20-30%) reduction of the resting $[Ca^{2+}]_i$. However, this may be unique to this cell system, as overexpression (by 3-4 fold) of PMCA1a in rat aortic endothelial cells did not alter basal $[Ca^{2+}]_i$ in these cells (62). Similarly, we have recently investigated stably transfected PC12 cells that either overexpressed exogenous hPMCA4b or were deficient in their endogenous PMCA4 (63). In both cases, no significant change of the resting Ca^{2+} level was seen in these cell lines compared to control cells (Michael L. Garcia, Yuriy Usachev, Stanley A. Thayer, Emanuel E. Strehler and Anthony J. Windebank, manuscript in preparation). Although the above examples are from non-neuronal cells, they illustrate the complexity of the link between a change in total PMCA expression and the resting intracellular Ca^{2+} level.

The different Ca^{2+} regulatory systems are obviously connected by complex feedback and cross-talk mechanisms whose existence and extent may be cell-type dependent. For example, overexpression of exogenous PMCA1a in rat aortic endothelial cells, or of PMCA4b in Chinese hamster ovary cells leads to compensatory changes (i.e., a downregulation) of the cells' endogenous SERCA pumps (62, 64, 65). An important signal that induces changes in PMCA expression and is involved in neuronal (and non-neuronal) differentiation is Ca^{2+} itself. Depolarization-induced transient elevation of $[Ca^{2+}]_i$ in cultured human IMR32 neuroblastoma cells resulted in a rapid change in the N-terminal alternative splicing pattern of PMCA2 as well as in an about 4-fold increase of PMCA2 mRNA and protein levels (66). Similarly, the expression of PMCA isoforms 1a, 2 and 3 was dramatically upregulated in rat neonatal cerebellar granule cells differentiating under membrane depolarizing conditions (67). Interestingly, however, PMCA4 was downregulated under these same conditions, suggesting that different PMCA isoforms are specifically and individually controlled by the depolarization signal. The primary signal inducing the observed changes in PMCA isoform expression is a rise in $[Ca^{2+}]_i$: Lowering extracellular Ca^{2+} or blocking L-type Ca^{2+} channels by nifedipine abolished the effect of depolarization on PMCA expression (67). Taken together, the above studies on the relationship between differentiation, Ca^{2+} signaling and changes in PMCA expression reinforce the emerging notion that different PMCA isoforms perform specialized and non-overlapping functions in different cells, and are not only involved in the maintenance of basal Ca^{2+} levels but are also active determinants of the downstream effects of Ca^{2+} signaling.

4.2. Hair cell adaptation

Although the knockout and PMCA2 mouse mutant studies discussed in sections 3.1. and 3.2. indicate the importance of the PMCA (specifically, of PMCA isoform 2) for the proper function of the inner ear, they do not address the specific contribution of the PMCA to the physiology of the ear. Mechanical stimuli applied to a hair bundle of the inner ear elicit electrical responses by regulating the opening and closing of mechanically sensitive transduction channels (68). Movement of the bundle toward its tall edge opens the transduction channel whereas movement toward its short edge closes the channel. During prolonged hair bundle deflection, transduction channel sensitivity is reset. This phenomenon, termed adaptation, is believed to be mediated by Ca^{2+} binding to calmodulin which in turn regulates the functioning of myosin 1-beta, the proposed molecular motor of adaptation (49, 69). Increasing the concentration of Ca^{2+} in the endolymph increases the rate and extent of adaptation. Due to the central role of Ca^{2+} in regulating adaptation, tight regulation of the endolymphatic as well as the intracellular Ca^{2+} concentration of hair cell stereocilia is critical. Recent evidence indicates that hair bundles may contain up to 2000 PMCA molecules per square micrometer of bundle membrane and that the PMCA is the principal calmodulin binding protein of the bundle (50). Moreover, pharmacological inhibition of PMCA activity leads to a large rise in resting intracellular Ca^{2+} and blocks the transduction-dependent outward current within hair bundles (50). Although unable to eliminate other forms of intracellular Ca^{2+} buffering, Yamoah *et al*. (50) concluded that the PMCA is one of the dominant factors controlling the concentration of Ca^{2+} in stereocilia (see figure 3A).

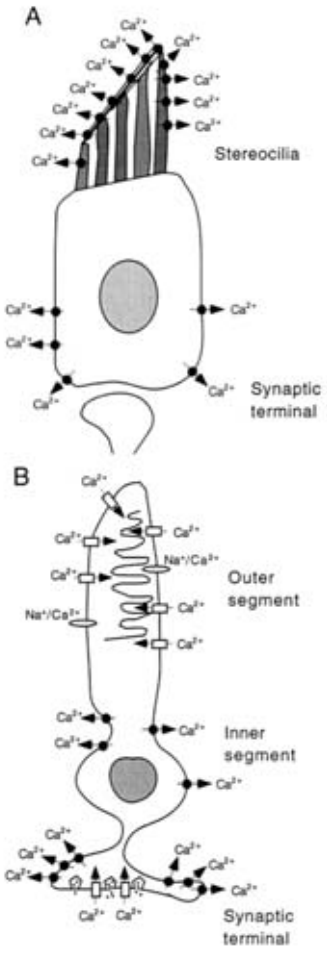

Figure 3. Schematic diagram of two types of sensory neurons emphasizing the specific subcellular localization of plasma membrane calcium ATPases. A. Scheme of a cochlear outer hair cell, where plasma membrane calcium pumps are highly concentrated in the stereocilia (darkly shaded) and are found at lower density in the basolateral membrane close to the synaptic terminals. The isoform in the stereocilia is PMCA2, whereas that in the basolateral membrane is likely different (probably PMCA1). The PMCAs are shown as black balls with an arrow indicating the direction of Ca^{2+} transport. For simplicity, all other transporters (e.g., Ca^{2+} channels) involved in transmembrane Ca^{2+} flux are not shown. B. Scheme of a rod photoreceptor cell with its distinct outer and inner segments and synaptic terminal region. The PMCAs are concentrated in the synaptic terminal and inner segment, whereas Na^{+}/Ca^{2+} exchangers (open ellipses) are present mostly in the outer segment. Ca^{2+} channels in the outer segment and synaptic zone are indicated as open rectangles. Note that the PMCAs are excluded from the active zone of neurotransmitter release (shown as black dots in synaptic vesicles), thereby helping to establish a standing Ca^{2+} gradient in the vicinity of the synaptic terminal. Also note that apical (e.g., stereociliar) and basolateral (synaptic terminal) PMCAs may correspond to different splice variants: the "a" form may be targeted to the stereocilia, and the "b" form to the synaptic region.

These conclusions have obviously been validated by the PMCA2 knockout and the mouse PMCA2 mutant studies. The underlying findings represent one of the most dramatic examples so far for the unique function of a specific PMCA isoform in handling Ca^{2+} dynamically in response to an extracellular signal (the mechanical displacement of the hair cell stereocilia following a sound pressure wave).

4.3. Transmitter release at photoreceptor synaptic terminals

Unlike in most other CNS neurons where brief pulses of Ca^{2+} influx through voltage-gated Ca^{2+} channels trigger pulses of neurotransmitter release, presynaptic calcium channels of photoreceptor terminals remain constantly open in the dark, allowing the tonic influx of Ca^{2+} and the consequent continual release of the neurotransmitter glutamate (70). Photoreceptors respond to light by hyperpolarizing and decreasing the rate of glutamate release. Voltage-gated calcium channels close in response to hyperpolarization, thereby decreasing the influx of Ca^{2+} ions. The constant influx of Ca^{2+} ions into the presynaptic terminal region in the dark requires that photoreceptors have an efficient Ca^{2+} extrusion system to prevent diffusion of Ca^{2+} into the soma and to ensure a rapid decrease in Ca^{2+} upon light stimulation (71). Two recent contributions have provided new and important information on the expression, localization and relative contribution to Ca^{2+} handling of the two major plasma membrane Ca^{2+} export systems in photoreceptors. Morgans *et al.* (71) compared the expression levels of the Na^{+}/Ca^{2+} exchanger and the PMCA in photoreceptors of the rat, tree shrew and goldfish retina. Immunofluorescence detected high levels of PMCA expression in the terminals of both rod and cone photoreceptors whereas the Na^{+}/Ca^{2+} exchanger was weakly expressed in cones and not at all in rods. Na^{+}/Ca^{2+} exchanger immunoreactivity was more abundant in the inner retina where strong staining of amacrine cells in the inner plexiform layer was observed. By contrast, PMCA staining was very high only in the synaptic areas, more dramatically so in the outer plexiform layer (region of phororeceptor synapses) but also in the inner plexiform layer where it was suggested to detect bipolar cell terminals (71). Using the same PMCA-specific antibody, Krizaj and Copenhagen (72) independently found that PMCA immunoreactivity was essentially confined to the synaptic regions in the tiger salamander retina. The strongest expression of PMCA was again found in the synaptic terminal region in the outer plexiform layer as well as in the basal region of the inner segment of both rods and cones (figure 3B).

The importance of the PMCA to the local regulation of intracellular Ca^{2+} in the photoreceptor inner segment was further demonstrated by electrophysiological experiments showing that blocking PMCA function by orthovanadate or ATP depletion inhibited Ca^{2+} extrusion. In contrast, replacement of extracellular Na^{+} with Li^{+} - which is known to inhibit Na^{+}/Ca^{2+} exchanger activity - had no effect on Ca^{2+} extrusion (71). Combining Ca^{2+} imaging in isolated, single photoreceptor cells with pharmacological interventions, Krizaj and Copenhagen (72) also convincingly showed that the outer and inner segments of

the photoreceptor cells are independent compartments with respect to their Ca^{2+} extrusion mechanism. Ca^{2+} extrusion from the outer segment occurs mainly via a Na^{+}/Ca^{2+} exchanger whereas Ca^{2+} efflux from the inner segment and synaptic terminal region is controlled by the PMCA (figure 3B). Distinct influx and efflux mechanisms as well as the restricted diffusion of Ca^{2+} thus contribute to the generation of separate Ca^{2+} regulatory compartments in the outer and inner segments of photoreceptors, allowing the functional uncoupling of sensory transduction (outer segment) and synaptic transmission (inner segment).

Interestingly, double-labeling experiments indicate that the active zone of the synaptic terminals is spatially distinct from the site of PMCA labeling. Whereas L-type Ca^{2+} channels are concentrated at the base of the terminals, the PMCA is concentrated along the sides and neck of each photoreceptor terminal; hence channels and pumps occupy strictly non-overlapping regions of the synaptic terminal (71) (figure 3B). This localization of the PMCA and the channels may lead to the formation of a standing Ca^{2+} concentration gradient in the vicinity of the synaptic zone and could ensure the maintenance of locally high Ca^{2+} required for tonic glutamate release while preventing large increases in nearby soma Ca^{2+} (71). The rate at which glutamate release can be terminated in response to light is determined by the speed of the reduction of synaptic Ca^{2+} and thus the local Ca^{2+} extrusion rate. Therefore, the PMCA, which determines the rate of Ca^{2+} extrusion at the synaptic terminals, may exert significant influence over the kinetics of synaptic transfer (71). It is not known yet which PMCA isoform(s) or splice variant(s) are responsible for the Ca^{2+} extrusion at photoreceptor terminals and inner segments. It is tempting to speculate, however, that a specific isoform and splice variant, possibly of the neuronal-specific PMCA2, is the major pump at these locations. The "b" splice form of PMCA2 which shows the highest Ca^{2+} pumping activity of all PMCAs (21) and has recently been shown to interact with synapse-associated PDZ proteins (27) is arguably the best candidate for this role.

5. ROLE OF THE PLASMA MEMBRANE CALCIUM ATPASE IN CALCIUM-MEDIATED CELL DEATH

Glutamate is one of the major excitatory amino acid neurotransmitters in the central nervous system (CNS) (73). The final common result of the activation of glutamate receptors is an increase in the intracellular Ca^{2+} concentration within the post-synaptic cell. The consequences of this rise in intracellular Ca^{2+} can be both positive and negative within a given population of neurons. For example, sufficient activation of ionotropic glutamate receptors within pyramidal cells of the CA1 region of the hippocampal formation is thought to lead to a persistent increase in synaptic strength known as Long Term Potentiation (LTP) (74). This activation of glutamate receptors leads to a rise in the intracellular Ca^{2+} concentration that is responsible for the induction of LTP. On the other hand, high concentrations of glutamate within the synaptic cleft can lead to prolonged and sustained activation of glutamate receptors. Over-stimulation of glutamate receptors and the resulting sustained intracellular Ca^{2+} overload may lead to cell death within vulnerable populations of neurons (75). This glutamate excitotoxicity is thought to be the final common pathway of acute injuries such as seizures or hypoxia, as well as of many neurodegenerative diseases such as Alzheimer and Huntington's disease (76-80). Excitotoxicity results in two forms of cell death, an acute and a delayed form (81). The acute form of cell death is a consequence of large increases in extracellular glutamate on ion fluxes within the neuron. The persistent activation of glutamate receptors results in the continued depolarization of the neuron and leads to increases in intracellular concentrations of Na^{+} and Cl^{-} (75, 81-83). The concomitant influx of water will result in cellular swelling and eventual cell lysis (81). Excitotoxicity can also lead to a delayed form of cell death which may occur days to weeks after an acute injury and might also be responsible for the observed loss of neuronal populations that is the hallmark of neurodegenerative diseases (78, 81, 84). Delayed cell death due to glutamate excitotoxicity results from the influx of extracellular Ca^{2+} (81, 85). Although the initial rise in Ca^{2+} may not be toxic to the cell, at later times a second rise or delayed Ca^{2+} deregulation occurs (86-88). The delayed increase in intracellular Ca^{2+} may result from mitochondria releasing sequestered Ca^{2+} back into the cytosol (89). However, relatively little evidence exists to explain which primary Ca^{2+} regulatory mechanism(s) is (are) affected during neuronal injuries resulting in the loss of Ca^{2+} homeostasis.

5.1. PMCAs as targets of free radicals

Excess intracellular Ca^{2+} can lead to delayed cell death in a variety of injury paradigms. Evidence indicates that excess intracellular Ca^{2+} can lead to the generation of free radicals within vulnerable neuronal populations which in turn induces Ca^{2+} dyshomeostasis (90). Prevention of free radical formation has been shown to prevent subsequent Ca^{2+} dyshomeostasis and neuronal cell death (90). Although Ca^{2+} dyshomeostasis has been linked to delayed cell death since 1985 (83), relatively little is understood about the effects of free radicals on Ca^{2+} homeostatic mechanisms. In 1993, Vincenzi and colleagues analyzed the effects of free radicals on the functioning of the PMCA (91, 92). Incubation of human red blood cells in the presence of ferrous sulfate and EDTA (Fe^{2+}/EDTA) or t-butyl hydroperoxide resulted in a concentration- and time-dependent inhibition of the PMCA (91-93). Furthermore, inhibition of PMCA by Fe^{2+}/EDTA was associated with cross-linking of membranous proteins. Free radical scavengers prevented both PMCA inhibition and membrane protein cross-linking (91-93). Evidence from these studies was the first indication that the PMCA may be a protein worthy of further investigation as an important candidate involved in Ca^{2+} mediated delayed neuronal death.

5.2. PMCA expression following seizures

Recent work from our own laboratory has attempted to further investigate the potential participation of the PMCAs in altered Ca^{2+} homeostasis following excitotoxic injury within a neuronal system. Isoform-specific changes in the expression of PMCAs 1, 2, and 3 in the rat hippocampus were analyzed utilizing *in situ*

hybridization following kainic acid (KA) injections (94). Decreased expression of mRNA corresponding to isoforms 1 and 2 was detected within the pyramidal cells of CA1 and CA3. Expression levels decreased 12 hours following KA injections and in some instances expression remained below control 72 hours following injections (94). As verified by histological staining, decreased PMCA expression preceded the onset of neuronal cell loss, which is inherent to KA injections, within the pyramidal cells of both CA1 and CA3 (M.G., unpublished observations). Interestingly, the most robust decrease in expression occurred specifically for PMCA2 within the granule cells of dentate gyrus 4 hours following injections (94). Expression of PMCA2 quickly rebounded and returned to control levels 24 hours following injections. In contrast to PMCA1 and PMCA2, no alterations in the expression pattern of PMCA3 mRNA were detected at any time point examined (94). The lack of KA effects on PMCA3 expression and the distinct time course of changes in PMCA1 and PMCA2 mRNA expression in different subregions of the hippocampus indicates that the early decreases in PMCA1 and 2 mRNA can not simply be explained by cell death or a generic response of all PMCA genes to status epilepticus. This is further supported by the finding that specific PMCA mRNA levels recovered to control levels at later time points in hippocampal subregions directly affected by seizures. The specific PMCA decreases reported by Garcia *et al*. (94) suggest that loss of Ca^{2+} homeostasis occurs within pyramidal cells during status epilepticus. Hours subsequent to increased neuronal activity the pyramidal cells experience a decrease in the two major PMCA isoforms (1 and 2) expressed in the brain (94). This may impair Ca^{2+} homeostasis to the point that over a prolonged period of time $[Ca^{2+}]_i$ is able to reach toxic levels.

Granule cells of the dentate gyrus are resistant to excitotoxic delayed cell death, yet the most dramatic decrease following KA induced seizures was observed for PMCA2 mRNA in the granule cells. However, this decrease was of a transient nature, occurring within 4 hours after seizure onset. By 24 hours after the insult the expression of PMCA2 mRNA had recovered to levels above control values (94). Thus, at times when pyramidal cells are undergoing delayed cell death, granule cells are expressing control or even above control levels of the major PMCA isoforms. This may allow these cells to maintain "normal" Ca^{2+} levels during times that are crucial to cell survival. A study looking at the distribution of the PMCA isoforms in the human hippocampus also found increased levels of PMCA2 (as well as of PMCA4) mRNA in the granule cell layer of the dentate gyrus, as well as in the CA2 pyramidal cell region (95). The latter region is known to be comparatively more resistant to excitotoxic cell death than the CA1 and CA3 regions (96); thus, the increased PMCA expression in this region may again point to the importance of the PMCAs in preventing Ca^{2+} dyshomeostasis following excitotoxic injury.

5.3. Effects of altering PMCA expression on cell survival

The studies showing direct effects of free radical damage and kainic acid-induced excitotoxicity on the PMCAs (91, 93, 94) beg the question of whether direct manipulations of PMCA levels may confer increased (overexpression) or decreased (downregulation) protection against Ca^{2+} mediated cell death on a neuron. Our own laboratory recently turned to a PC12 cell model to further address the role of the PMCA in Ca^{2+} mediated cell death and the potential mechanism of growth factor protection from Ca^{2+} mediated cell death. Utilizing antisense strategies, similar to those reported by Vanaman and colleagues (60), we suppressed the expression of endogenous PMCA4 within PC12 cells. PC12 cells were also analyzed that were engineered to overexpress the human PMCA4b isoform. Utilizing the calcium ionophore A23187 to induce calcium mediated cell death, the survival rates of cells were analyzed with and without alterations in their PMCA4 expression (63). PMCA4-depleted PC12 cells (expressing about 35% of the PMCA4 in control cells) were considerably more vulnerable to Ca^{2+} mediated cell death than control cells whereas overexpressing cells (containing about 1.5 fold the amount of PMCA4 of controls) were less vulnerable than controls (97). A surprising result was found when PC12 cells with altered PMCA4 expression were incubated in the presence of both nerve growth factor (NGF) and ionophore, and then examined for their survival rates. If the PMCA were a necessary component of the mechanism by which NGF protects cells from Ca^{2+} mediated cell death, PMCA-depleted PC12 cells should be expected to be less protected and overexpressing cells better protected (or at least equally well protected) than control cells. In fact, the opposite was observed: Depletion of PMCA4 afforded better protection whereas cells that overexpressed PMCA4 were less protected than controls against Ca^{2+} mediated cell death in the presence of NGF (97). Taken together, the data indicate that the PMCAs are critical to PC12 cell survival and are involved in the maintenance of Ca^{2+} regulation in the face of a Ca^{2+} insult; however, these pumps (at least PMCA4) do not seem to be a necessary component of the mechanism by which NGF protects the PC12 cells from Ca^{2+} mediated cell death (98). Clearly, compensatory changes in other Ca^{2+} regulatory systems (buffering proteins, channels, SERCA pumps) may complicate the interpretation of these data (see also section 4.1); however, the pronounced changes in Ca^{2+} handling that result from the selective overexpression or downregulation of a given PMCA isoform support the overall notion that each PMCA isoform fulfills a specific role in the long-term and dynamic regulation of intracellular Ca^{2+}.

6. CONCLUSIONS AND PERSPECTIVE

The past few years have seen a large growth in research into the many facets of the isoform distribution, regulation, and cellular function of the PMCAs. Many of these observations have extended the role of the PMCA from its status as an important but somewhat "boring" Ca^{2+} extrusion system responsible solely for maintaining basal intracellular Ca^{2+} concentrations. Recent studies indicate that the PMCAs are critical regulators of intracellular Ca^{2+}, but not only in a slow, tonic way acting over long time periods but also as active participants in shaping Ca^{2+} signals in a spatially and temporally defined manner. The PMCAs are necessary for

the proper development, functioning and survival of neuronal systems. Different PMCA isoforms and their splice variants fulfill specific, non-redundant functions in these processes, and they are adapted to perform specific roles in different cells and even within a single cell.

Ca^{2+} signaling and Ca^{2+} mediated physiological responses are highly compartmentalized in many cells - especially in the highly asymmetric neurons - and must occur with different spatial and temporal regulation. For example, the rise in Ca^{2+} in the stereocilia of a hair cell following positive deflection of hair bundles and transient opening of the elctromechanical transduction channel is important for the feedback adaptation of the channels and is confined to the hair bundles. By contrast, Ca^{2+} elevations at the basolateral membrane in the vicinity of afferent synapses are important for transmitter release and must be spatially and temporally insulated from other Ca^{2+} mediated events. This is particularly obvious in the sensory hair cells or in the photoreceptor rods and cones (discussed in sections 3 and 4) where distinct compartments with different Ca^{2+} influx and efflux properties are clearly separated. Thus, the molecular components of different Ca^{2+} signaling domains must be tightly coupled and properly organized into Ca^{2+} "signalosomes" at specific cellular locations. Different PMCA isoforms and splice variants are likely targeted to specific cellular regions where they become part of such local Ca^{2+} signalosomes.

The data from the PMCA2 knockout and the PMCA2 mutant mice indicate that this isoform is the major PMCA in the hair bundles of auditory and vestibular hair cells and that its function is crucial to normal hearing and balance. Furthermore, other PMCA isoforms such as PMCA1 - which may be expressed in the same cell - can not compensate for a decrease or the absence of PMCA2. It is possible and even likely that different splice variants of PMCA2 are involved in Ca^{2+} regulation at different locations in the hair cells. Recent evidence has shown that one of the major C-terminal splice variants (the "b" form) of the PMCA can interact with PDZ domain containing signaling and scaffolding proteins that may anchor or target the pump to the basolateral or dendritic/postsynaptic membrane (26, 27). The other major splice variant (the "a" form) may well contain information that localizes it to a different (e.g., an apical) membrane region. It is possible, therefore, that one splice variant of a given PMCA isoform (e.g., PMCA2a) is exclusively responsible for Ca^{2+} extrusion in a cellular compartment such as the hair bundles, whereas another variant (e.g., PMCA2b) is involved in clearing Ca^{2+} from the basal membrane in the vicinity of synapses. Future studies using selective gene knockout (or "knock-in") strategies to disable specific alternative splice pathways of a given PMCA gene will hopefully shed light on these intriguing possibilities.

As these avenues of research into the role of different PMCAs in Ca^{2+} signaling in neuronal systems are relatively new, we have only begun to see the tip of the iceberg. Prospects for future discoveries that will further extend and better delineate the role of the diverse PMCA isoforms in regulating intracellular Ca^{2+} are now brighter than ever.

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