**[Frontiers in Bioscience 7, d53-84, January 1, 2002]**

# THE ROLE OF PLASMA MEMBRANE $CA^{2+}$ PUMPs (PMCAs) IN PATHOLOGIES OF MAMMALIAN CELLS

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## 1. ABSTRACT

The biochemical function of the plasma membrane calcium ATPases (PMCAs) is the extrusion of cytosolic $Ca^{2+}$ from the cell. Although this general function is well documented, the role of the complex isoform diversity and especially the contribution of specific isoforms to pathological conditions is less well understood. No human disease has been linked to a defect in any of the four PMCA genes. Nevertheless, isoforms do not have redundant functions, as shown by the indispensable role of PMCA2 demonstrated in transgenic mice. This review summarizes the results of recent analysis of the PMCA dysregulation in diseased cells or model systems of pathological conditions, including both acute disorders like hypoxia/ischemia and seizure, and slowly progressing dysfunctions like Alzheimer‘s disease, hypertension, diabetes and aging. Abnormalities in PMCA or its regulators have been described in various organs, reflected in changes of expression levels or in modifications or proteolysis of the PMCA protein. Changes of PMCA function are often detected in cell types different from the specific type involved in the pathology, pointing to more general defects. Examples are erythrocytes in diabetes and blood platelets in hypertension. The changes suggest the significance of PMCA in $Ca^{2+}$ homeostasis both in excitable and non-excitable cells.

## 2. INTRODUCTION

The recent extensive progress in the elucidation of the structure and function of Plasma Membrane $Ca^{2+}$-transport ATPases (PMCA) has been summarized in several excellent reviews, including (1, 2). PMCAs form a subfamily of the P-type $Ca^{2+}$-transport ATPases, that utilize the energy of the hydrolysis of ATP to drive uphill translocation of $Ca^{2+}$ across the membrane, transiently forming a phosphorylated intermediate during the transport cycle. The main structural property discriminating PMCAs from related subfamilies like the SERCAs of the internal $Ca^{2+}$ stores is the presence of an extended C-terminal tail forming an autoinhibitory domain and containing a calmodulin-binding site. Binding of $Ca^{2+}$-calmodulin relieves the inhibition, providing a mechanism for regulation of PMCA activity by the cytosolic $Ca^{2+}$ concentration itself. PMCAs form the sole ATP-driven $Ca^{2+}$-extrusion system of the plasma membrane. However, in most cell types they are assisted in their function by the $Na^{+}$-$Ca^{2+}$ exchangers that catalyze the uphill extrusion of $Ca^{2+}$ at the expense of the energy delivered by the downward counter-transport of $Na^{+}$. Exceptions are erythrocytes of most mammalian species, and hair cells of the auditory and vestibular system that solely rely on PMCAs for $Ca^{2+}$ extrusion. In general, the $Na^{+}$-$Ca^{2+}$ exchange system seems to have a higher transport capacity but a lower affinity for $Ca^{2+}$.

PMCAs come in a great number of different isoforms. The reader is referred to recent reviews for a detailed survey (2). PMCAs are encoded by four genes, designated ATP2B1, ATP2B2, ATP2B3 and ATP2B4 in the human genomic database, encoding respectively PMCA1, PMCA2, PMCA3 and PMCA4. Alternative processing of the primary transcripts further increases the isoform diversity to at least twenty. This great diversity is a complicating factor in the study of the effect of pathophysiological conditions on the expression of PMCAs. Moreover, alternative processing of the PMCA mRNA occurs at sites that correspond to positions in the translated sequence that are crucial for regulation of the $Ca^{2+}$ pump activity, probably resulting in a great diversity at the functional level as well (1, 2). PMCA1 and PMCA4 are almost ubiquitously expressed and are thus considered as housekeeping isoforms. PMCA2 and PMCA3 show a cell-type specific expression. For some isoforms also a specific subcellular localization in polarized cells has been demonstrated.

Alternative splicing affects cytosolic domains. The rearrangement occurs at two sites, designated site C (close to the C-terminus and located in the calmodulin-binding domain) and site A, located in the domain between transmembrane segments 2 and 3. Site C affects the binding of calmodulin and the susceptibility to phosphorylation by protein kinase A and by protein kinase C. The C-termini of some isoforms contain a PDZ-binding domain. Site A affects the sensitivity of PMCA to the stimulatory effect of negatively charged phospholipids (3). Thus PMCA activity is able to respond in a cell-type specific and in a great variety of ways to changing physiological or pathological conditions.

Up till now no human disease has been linked to a defect in any of the PMCA genes. In mice, however, genetic defects in the gene encoding PMCA2 demonstrate that this isoform is indispensable for hearing and balanced movements. PMCA is expressed at high levels in the outer sensory hair cells of the organ of Corti. PMCA isoforms other than PMCA2 apparently cannot compensate for the loss of PMCA2 from these cells (4, 5, 6). In most cell types, however, non-genetic alterations of PMCA activity may be an important factor contributing to pathological conditions, considering the central role of PMCAs in the $Ca^{2+}$ extrusion pathway and the demonstrated involvement of $Ca^{2+}$ overload in the onset and progress of cellular dysfunction. In order to throw some light on the complexity of PMCA alterations in relation to disease, it is necessary to follow several distinct lines of investigation, each with its specific scientific questions and specific experimental approach. In the following overview we will make a distinction between studies at the organ level and at the cellular level, studies on changes in expression of PMCAs and on functional activity, experimental induction of gross alterations such as hypoxia/ischemia, and more specific modifications of the experimental conditions. Taken together, the findings indicate that changes in PMCA activity may contribute at least to the progress and the severity of several pathological conditions.

## 3. CONSEQUENCES OF LOSS OF FUNCTIONAL PMCA GENES

The lack of known human pathologies linked to defects in any of the four PMCA genes could be indicative of a high degree of redundancy of the functions of each of the gene products. However, the great isoform diversity is found in all vertebrates, and even the nematode *Caenorhabditis elegans* expresses three PMCA genes (7). Such conservation strongly suggests an evolutionary pressure to preserve this diversity because of the requirement of isoform-specific properties for optimal functioning of the cell and the organism. Because of the lack of specific PMCA inhibitors, further proof will require the study of genetically modified animals, or the technique of specific mRNA elimination (antisense or RNAi) on cultured cells or model organisms. The application of these strategies has already given some striking examples of the indispensability of specific PMCA gene products.

Transgenic PMCA2-deficient mice exhibit an unsteady gait, have difficulties in maintaining balance and have hearing defects. The $Ca^{2+}$ extrusion from hair bundles of sensory cells of the inner ear is catalyzed solely by PMCA pumps, the ionic composition of the

endolymph being incompatible with $Na^+$ dependent $Ca^{2+}$ extrusion by the $Na^+$-$Ca^{2+}$ exchanger. By immmunoanalysis, Crouch and Schulte (8) have documented the distribution of PMCA in the adult and developing gerbil cochlea. Remarkable changes occur in the expression pattern of different isoforms in the developing cochlea between PMCA1 and PMCA2 supporting the notion that PMCA isoforms perform specific and specialized functions (9). Immunohistological analysis has revealed that PMCA protein is also localized to the endolymph-secreting epithelial cells and participates in the regulation of the $Ca^{2+}$ concentration in the endolymph (10).

In general, PMCA2 exhibits a highly restricted tissue distribution, suggesting it serves more specialized physiological functions than some of the other isoforms. In line with the PMCA2 knockout experiments, three different spontaneous mutations in the PMCA2 gene have been identified, occurring in two variants of „deafwaddler“ and one „wriggle mouse Sagami“. Analysis of heterozygous deafwaddler mice mutants has shown that PMCA2 is important for the outer hair function mainly at high frequencies (11). Thus, the data strongly argue for a specific role of PMCA2 in the ear and against the general redundancy of the PMCAs (2). For a comprehensive overview the reader is referred to reviews by (9, 12, 13).

## 4. ALTERATIONS OF PMCA PROTEIN EXPRESSION LEVELS

The proper cellular response to external and internal signals includes changes in the expression level of molecular components of different $Ca^{2+}$-regulatory pathways. PMCA isoform expression and their alterations in pathological conditions due to interactions on the transcriptional level have only recently begun to be elucidated**.** Changes in the mRNA levels as well as in alternative mRNA processing have been described in response to a variety of factors, among them $Ca^{2+}$ itself, vitamin D and steroids. In addition to their well known transcriptional effects, steroids have also been shown to have short-term effects on PMCA function. This section is restricted to changes observed in well controlled experimental conditions. Many other cases of altered expression levels will be described in later sections on pathological situations or disease-related conditions. The cause of these changes is often not well established.

### 4.1. Role of PMCA in growth and differentiation

#### 4.1.1. Neuronal cells

Calcium metabolism undergoes considerable changes during cellular differentiation. In neurons, differentiation induces up-regulation of calcium channels which help in the triggering of $Ca^{2+}$-dependent events (14). More recent evidence shows that also the PMCA expression is increased in parallel, probably reflecting the greater demand for $Ca^{2+}$ extrusion. Experiments have been conducted *in vivo* and on neuronal cells in culture, particularly cerebellar granule cells, PC-12 or the derived PC-6 cells and neuroblastoma cell lines.

PMCA2 and PMCA3 are neuron-specific isoforms. During neuronal differentiation, up-regulation of these isoforms appears to be a general phenomenon, irrespective of the *in vivo* or *in vitro* situation, or of the differentiation-inducing stimulus, as will be shown below. Changes of PMCA1 and PMCA4 are less pronounced and more variable. Although it is difficult to prove a causal link between PMCA activity and differentiation, additional experiments involving manipulation of PMCA activity, including work on non-neuronal cells, may suggest such a connection.

PMCA2, PMCA3 and to a lesser extent PMCA4 are up-regulated during the development of the cerebellum (15). Similarly, rat cerebellar granule cells in culture up-regulate PMCA activity when they are induced to differentiate, either by chronic exposure to $K^+$-depolarizing solution or to NMDA receptor agonists, treatments that both cause a sustained increase of $[Ca^{2+}]_i$. PMCA2 and PMCA3 are strongly up-regulated whereas a brain-specific splice variant of PMCA4, PMCA4CII, is down-regulated (15). Because this isoform contains a PDZ-binding domain, its specific loss may reflect a requirement for removal of PMCA activity at specific sites in differentiated cells. Guerini *et al.* (16) have demonstrated that the down-regulation of PMCA4CII is mediated by activation of the $Ca^{2+}$-calmodulin dependent protein phosphatase calcineurin. Calcineurin is also important in the control of the expression levels of the $Na^+$-$Ca^{2+}$ exchanger NCX2 and of $IP_3$ receptor type 1, but does not affect the up-regulation of PMCA2 and PMCA3. A common mediator is probably $[Ca^{2+}]_i$ itself because decreasing extracellular $Ca^{2+}$ or blocking $Ca^{2+}$ channels inhibits the responses at the level of the PMCAs.

In another model system, induction of differentiation of neuroblastoma IMR-32 cells by cyclic AMP similarly up-regulates PMCA2 and PMCA3. PMCA4 is increased as well. Concomitantly, the recovery of $Ca^{2+}$ transients in the presence of a SERCA blocker is accelerated, indicating a faster $Ca^{2+}$ efflux (17).

Although PMCA1 is not a neuron-specific isoform, the inhibition of PMCA1 expression by the antisense technique in PC-6 neuronal cells in culture leads to a loss of nerve growth factor (NGF)-mediated neurite extension. This loss is associated with an elevation of glucocorticoid responsiveness (see also 4.2), causing down-regulation of $\alpha_1$ integrin (18). These effects appear to be independent of any alteration of the cytosolic $Ca^{2+}$ concentration since the basal $[Ca^{2+}]_i$. or size of $[Ca^{2+}]_i$. transients were not significantly affected (19). The authors interpret these results as evidence for a role of specific PMCA isoforms that is more subtle and complex than simply removing $Ca^{2+}$ following a $Ca^{2+}$-mediated signal.

In addition to its effect on PMCA gene expression, elevation of [Ca2+]i also affects the splicing pattern of the mRNA. In the neuroblastoma cell line IMR-32, depolarization results in an up-regulation of PMCA2 and in a change of its N-terminal splicing pattern (20).

#### 4.1.2. Endothelial cells and smooth-muscle cells

In endothelial cells, the PMCA1 gene is up-regulated by agonist- and tissue-specific signalling pathways. Protein kinase A and protein kinase C have been implicated in the increase of PMCA1 expression. The regulation of this response is dependent on transcriptional activation of the PMCA1 gene via a core segment of the gene promoter (-442 to + 169) (21).

Interestingly, the PMCA1 promoter has recently been demonstrated to possess two binding sites for the transcription factor c-myb. Binding of c-myb represses transcription of PMCA1 in vascular smooth muscle cells at the $G_1$/S cell cycle interface (22). C-myb is a transcription factor vitally important for the control of cell proliferation in conditions such as atherosclerosis and restenosis and it is also involved in the regulation of apoptosis by modulation of $[Ca^{2+}]_i$. The reduction in c-myb activity inhibits cell cycle progression and decreases intracellular $Ca^{2+}$ concentrations. The effects are largely mediated by an increased rate of $Ca^{2+}$ extrusion, which is associated with increased levels of PMCA1 mRNA and protein, in parallel with lower but similar changes of PMCA4 mRNA. Conversely, overexpression of PMCA1 resulted in reductions of $[Ca]_i$, $G_1$ to S transition and rate of cell proliferation (23).

#### 4.1.3. Muscle cells

Overexpression of PMCA4 in the myogenic cell line L6 accelerates differentiation (24). This effect is in line with the anti-proliferative effect described above for vascular smooth cells. In contrast, overexpression of PMCA in the heart of transgenic mice stimulates growth of isolated cardiac cells in response to fetal calf serum or adrenergic agonists and attenuates induction of gene expression in response to endothelin 1 (25, 26).

#### 4.1.4. $Ca^{2+}$ transporting epithelia

Vitamin D-dependent $Ca^{2+}$ transporting tissues express PMCA1b as the major isoform. Pannabecker *et al.* (27) first showed that $1,25(OH)_2$ vitamin $D_3$ up-regulates the plasma membrane $Ca^{2+}$-pump protein in the intestine. Because nuclear PMCA mRNA was increased, the authors attribute the effect to an increased rate of transcription. Recently, however, it has been shown that post-transcriptional effects contribute to the up-regulation of PMCA1 (see 4.2) and that any positive regulatory vitamin D response element must lie outside the core promoter (28).

Taken together, all these results suggest that the function of PMCAs is not restricted to simple prevention of calcium overload, but may be extended to an active involvement in the fine tuning of $[Ca^{2+}]_i$ with potentially important consequences for cellular functions such as growth (18).

### 4.2. Effects of steroids

The brain is a site of extensive synthesis and metabolism of steroid hormones such that the accumulation of steroids appears to be, at least in part, independent of adrenal or gonadal sources.

Low levels of corticosteroids, mainly glucocorticoids, are important factors for learning and memory due to their genomic actions in the hippocampus. Chronically high levels of corticoids have profound effect on hippocampal structure and function and can even result in irreversible neurodegeneration. In a screen for genes repressed by high doses of glucocorticoids in rat hippocampus, the PMCA1 message has been picked up (29). Confirming this result, repression of PMCA1 by corticosterone was also observed in cultured hippocampal neurons, but only when the cells were in the differentiated state.

Because corticosteroids are mediators in the response to stress, Bhargava *et al.* (29) also investigated the effect of stress on PMCA1 expression. These effects were more complex, suggesting that corticosteroids are only one pathway in the PMCA response to stress. In intact animals cold stress itself repressed expression of PMCA1 in the hippocampus, whereas PMCA1 in other brain regions did not change. In adrenalectomized animals, cold stress even increased hippocampal PMCA1 expression. Defects in the balance between glucocorticoid-mediated and non-glucocorticoid-mediated effects on PMCA1 expression may have adverse effects on neuronal function and ultimately result in irreversible neuronal damage (29).

The PMCA-glucocorticoid relation is confirmed by the converse experiment already described above (see 4.1.1.), demonstrating that the loss of PMCA1 by using antisense technology in PC-6 cells is associated with increased glucocorticoid receptor transactivation (18).

Several neuroactive steroids have been shown to affect the activity of plasma membrane $Ca^{2+}$-stimulated ATPase activity independent of transcriptional effects. In dog synaptosomes, testosterone stimulated the activity whereas progesterone had an opposite effect. In rat brain neuronal membrane preparations or purified rat $Ca^{2+}$-ATPase, an increase of the activity of $Ca^{2+}$-ATPase was demonstrated after a short-time incubation with physiologically relevant concentrations of pregnenolone, estradiol, testosterone and dehydroepiandrostendione, which suggests that local steroid synthesis could allow permanent $Ca^{2+}$-independent non-genomic $Ca^{2+}$-ATPase regulation (30).

## 5. MODIFICATIONS OF PMCA PROTEINS

It is generally accepted that oxidative stress, initiated mainly by reactive oxygen/nitrogen species [RO(N)S], contributes as a pathogenic factor to several pathologies including ischemia/reperfusion injury (IRI), and inflammatory and degenerative diseases. The disruptive action of RO(N)S involves membrane lipid peroxidation, membrane protein modification, DNA damage and formation of advanced glycosylation products. Each of these events may cause alterations of membrane structure and function, including membrane fluidity and activity of structural proteins. The changes of redox balance often lead to activation or silencing of genes encoding transcription factors such as AP-1 and NF-κB, antioxidant defense enzymes, and structural proteins which may modulate signal transduction cascades and redirect gene expression in eukaryotic cells (31).

### 5.1. Functional modifications of PMCA induced by oxidative stress

The effect of oxidative stress on membranes is known to involve modifications of both protein and lipid. Because the activity of the plasmalemmal $Ca^{2+}$-transport ATPase not only depends on the integrity of the pump protein itself but also on the lipid composition of the membrane, it is often difficult to find the exact mechanism responsible for oxidative impairment of $Ca^{2+}$ pump activity. Evidence for both types of mechanisms has been obtained, depending on the oxidative agent and on the cell type.

The first indications of PMCA protein as a possible target of free radicals came from studies on erythrocytes which showed that incubation of red blood cells in the presence of non-heme iron, ferrous sulfate and EDTA, or t-butyl hydroxyperoxide resulted in a concentration- and time-dependent inhibition of PMCA (32 - 34). The inhibition was associated with cross-linking of membrane proteins. Free-radical scavengers prevented both PMCA inhibition and protein cross-linking.

Clear evidence for a direct effect on the $Ca^{2+}$ pump protein itself has been obtained for $Ca^{2+}$ transport inhibition in erythrocytes by activated neutrophils (35). The inhibition is caused by superoxide produced by neutrophils which is converted to other radicals by a Fenton-type reaction. By using an antibody to the $Ca^{2+}$ pump protein, it was shown that the inhibition was accompanied by protein fragmentation. A contribution of lipid peroxidation was unlikely. Pump protein degradation has also been demonstrated during exposure of erythrocytes to peroxynitrite (36).

A direct inhibitory efffect on the erythrocyte $Ca^{2+}$-transport ATPase is also seen with 4-OH-2,3-*trans*-nonenal (HNE), an aldehyde product of fatty acid oxidation, although this effect is accompanied by an increased passive $Ca^{2+}$ permeability, indicating an effect on lipids as well. The inhibition of the $Ca^{2+}$ pump is due to the depression of the basal as well as the calmodulin-stimulated activity (37). Pretreatment of calmodulin itself with HNE did not impair its ability to stimulate the $Ca^{2+}$-ATPase activity. Detailed analysis of the kinetics of $Ca^{2+}$ pump inhibition demonstrated a reduction of $V_{max}$ as a result of a direct effect on the enzyme, probably by irreversible modification of functionally important sites on the enzyme molecule (38). The effect of HNE provides an example of the complexity of oxidative damage due to additional mechanisms that may be involved. It has been shown that HNE is present in oxidized low density lipoproteins (LDL), which are found in atherosclerotic lesions (39). Thus, together with the modification of low density lipoproteins (LDL), the $Ca^{2+}$ pump and other proteins need to be considered as additional sites of action of HNE in atherogenesis or other radical-generated pathologies. Results which further support this hypothesis have been obtained from exposure of platelet plasma membranes to $Cu^{++}$-oxidized LDL as an inhibitory agent. Oxidized LDL but not native LDL inhibited the $Ca^{2+}$-ATPase activity in parallel with a massive increase of $[Ca^{2+}]_i$. This treatment however did not alter membrane fluidity, excluding gross changes of the membrane environment (40). The inhibitory concentration of oxidized LDL used in the study was considerably below the physiological level of circulating LDL, indicating that platelet $Ca^{2+}$-ATPase may also be inhibited *in vivo*. Thus, modifications of $Ca^{2+}$-ATPase may contribute to elevated $[Ca^{2+}]_i$ and platelet activation in such pathologies as atherosclerosis and hypertension.

Other reactive molecules that have been shown to inhibit the $Ca^{2+}$-ATPase of erythrocytes are amino acid peroxides and reactive nitrogen species (RNS), such as NO (nitric oxide) or $ONOO^-$ (peroxynitrite) (41). These substances are powerful oxidants, which can react with unsaturated fatty acids and with amino acid residues of proteins. Erythrocyte membranes exposed to peroxynitrite present aggregation and nitration of proteins, changes in protein organization and inactivation of the $Ca^{2+}$-ATPase activity. Neuromelanin, a product of dopamine metabolism in the brain, may act as a physiological protector against peroxynitrite as it inhibits nitration of tyrosine and oxidation of tryptophan residues in the erythrocyte $Ca^{2+}$-transport ATPase (42, 43).

The peroxynitrite-induced protein degradation pattern of the erythrocyte $Ca^{2+}$-ATPase is also seen in erythrocytes of asphyxiated newborn patients. The perinatal hypoxic-ischemic encephalopathy (asphyxia) is a major cause of childhood neurological deficits and it is suggested that cooperative action of RO(N)S and excitatory amino acids are involved in the etiology of the injury. The neuronal cell damage is not restricted to neurons but can also be detected in blood cells. An enhanced lipid peroxidation has been detected in

erythrocyte ghosts of asphyxiated newborns in parallel with an increased degradation of $Ca^{2+}$-pump protein and a concomitant depression of $Ca^{2+}$-ATPase activity to 50 %. Moreover, the activity of the $Ca^{2+}$-ATPase in asphyxiated membranes was stimulated by calmodulin to a lesser degree than that of normal membranes. Since administration of peroxynitrite to purified $Ca^{2+}$-ATPase from human erythrocytes caused a time-dependent protein cleavage, it is suggested that this membrane is a primary target of asphyxia-induced damage, in part mediated by ROS. The impairment of $Ca^{2+}$-ATPase in erythrocytes thus might destabilize cellular $Ca^{2+}$ homeostasis and could contribute to hypoxia-induced damage to the cell (36).

In alveolar macrophages, oxidative stress has been shown to cause alterations in cellular morphology and signalling pathways during respiratory burst and prolonged inflammatory reactions. An early alteration to the plasma membrane is the translocation of annexin VI as a result of ATP depletion and oxidative modification of membrane lipids and proteins. During respiratory burst, $[Ca^{2+}]_i$ is elevated. In parallel, a dysfuction of plasma membrane $Ca^{2+}$-ATPase caused by hydroperoxides has been proposed as a potential mechanism to explain the cellular reactions (44). Similarly, incubation of hepatic basolateral plasma membrane with S-nitroso-N-acetyl-penicillamine (a NO donor) or 3-morpholinosydnonimine (a peroxynitrite donor) decreased the $Ca^{2+}$-ATPase activity in a manner dependent on the concentration of the liberated NO or peroxynitrite. Both compounds likely inhibit the ATPase activity by oxidation of thiol groups of the enzyme (45).

Disturbances in $Ca^{2+}$ homeostasis play a particularly important role in brain damage and in a number of processes that have been implicated in $Ca^{2+}$-related pathogenesis, including brain aging, ischemia/anoxia, oxidative stress, and several neurodegenerative diseases. The CNS with its high rate of oxygen consumption and increased content of polyunsaturated fatty acids is a tissue especially vulnerable to oxidative stress. $Ca^{2+}$-ATPase from cortical synaptosomes shows a diminished activity following ascorbate/iron induced oxidation (46), which is accompanied by protein cross-linking and lipid peroxidation. This suggests that inhibition is the result of both lipid peroxidation and enzyme protein modification. $[Ca^{2+}]_i$ is increased in the oxidative condition if $Ca^{2+}$ is present in the extracellular medium. The membrane potential is changed only slightly, suggesting that the integrity and permeability of the synaptosomal membrane is preserved. The defects in $Ca^{2+}$extrusion (via PMCA) and/or sequestration (via SERCA) might be responsible for the alteration in calcium homeostasis as a consequence of lipid peroxidation (46). Exposure of PMCA to peroxyl radicals, hydrogen peroxide and ONOO´ *in vitro* led to the significant loss of PMCA $Ca^{2+}$-ATPase activity, kinetically expressed in a decrease of $V_{max}$ without significant changes in $Ca^{2+}$ affinity (47). Moreover, the direct measurement of $Ca^{2+}$ transport was a more sensitive indicator of oxidative damage to the enzyme than the $Ca^{2+}$-activated hydrolysis of ATP. Peroxyl radicals and hydrogen peroxide act quite non-selectively, whereas the effect of $ONOO^-$ is more selective and specifically modifies residues such as tyrosines, tryptophanes, and cysteines. Exposure of synaptosomes to oxidative stress led both to the introduction of carbonyl groups into protein and to induction of structural changes such as protein aggregation detected by anti-PMCA antibodies. The effect is partially reversed under reducing conditions. The aggregation is probably the result of bityrosine formation, unfolding of the protein and increased hydrophobic interactions between PMCA molecules (47).

## 6. PMCA ALTERATIONS IN CALCIUM-RELATED CELL TOXICITY

Overstimulation of glutamate receptors leads to excitotoxic damage of selectively vulnerable neurons, resulting in necrotic or apoptotic cell death (31, 48). This glutamate excitotoxicity is thought to be a final common pathway of acute injuries such as seizure and ischemia (stroke), as well as of many neurodegenerative diseases. The sustained elevated levels of glutamate may result in delayed cell death by increased permeability of mainly NMDA and AMPA receptors to $Ca^{2+}$. Glutamate toxicity depends also on the generation of oxygen free radicals and NO (31) which have been shown to alter the functioning of ion-transporting ATPases including PMCA pumps (47, 49). Although the initial rise of $Ca^{2+}$ may not be acutely toxic, at later times a delayed $Ca^{2+}$ deregulation occurs. However, little evidence exists to explain the role of PMCA pumps or other $Ca^{2+}$ regulatory mechanisms in the glutamate-induced cell damage.

Intracellular $Ca^{2+}$ overload caused by excessive glutamate release and concomitant generation of free radicals are thought to play a prominent role as triggering factors in the pathogenesis of brain hypoxic (anoxic), hypo- aglycemic and ischemic-reperfusion injury (IRI) (50). The oxygen supply to part of the brain can be cut off by a loss of blood flow (ischemia), owing to cardiac arrest or a clot occluding a blood vessel, or by insufficient oxygen in the blood (hypoxia), as in perinatal asphyxia or poisoning with CO. All those pathologies including substrate withdrawal (hypo- or aglycemia) quickly lead to dissipation of transmembrane gradients, membrane depolarization and to an acid shift of the intracellular pH. Numerous energy-dependent homeostatic mechanisms are necessary to support normal neuronal function. In addition, increased influx of extracellular $Na^+$ and inhibition of the $Na^+$-$K^+$-ATPase and other ion pumps including PMCA due to ATP depletion can exacerbate the effects of hypoxia on the neuronal cells (51). The brain is especially vulnerable to free-radical damage because of its high

oxygen consumption, abundant lipid and iron content, and relative paucity of antioxidants compared with other organs. Because the capacity of cerebrospinal fluid to bind iron is poor, the iron release by hemorrhage, or its mobilization from ferritin by acidosis may trigger generation of RO(N)S.

Among the effects of ischemia/reperfusion, lipid peroxidation, modification of the phospholipid profile and the accumulation of aldehydes such as HNE are the most prominent. Protein oxidation such as amino acid modification, fragmentation and cross-linking might lead concomitantly to secondary accumulation of modified proteins (52, 53, 54). In addition, the redox status altered by disease may modify protein degradation (55). Derivatives of nitrogen such as peroxynitrite may induce nitration of tyrosines in proteins, thereby preventing their phosphorylation and causing interference with cellular signalling (56). Oxidative alterations of functional proteins and lipids after IRI could contribute to the disturbance of neuronal ion gradients including $Ca^{2+}$ homeostasis (57) and consequently might lead to neuronal cell death (31). From *in vitro* experiments it is known that effects of RO(N)S are rather unspecific. However, radicals strongly inhibit important enzymes regulating neuronal ion homeostasis such as $Na^+$-$K^+$-ATPase (58, 59, 60) and the SERCA $Ca^{2+}$ pump (57). Several lines of evidence suggest that also the PMCA $Ca^{2+}$ pump is one of the target proteins for RO(N)S (for recent review see (61)).

The relative contribution of the mechanisms involved in the clearance of intracellular $Ca^{2+}$ has been shown to be dependent on the $Ca^{2+}$ concentration as well as on the neuronal cell type. Only a limited number of papers deal with the subcellular localization of PMCA transporters in neurons. Biochemical and immunocytochemical studies on chick ciliary ganglion have clearly shown that both PMCA and $Na^+$-$Ca^{2+}$-exhanger are present in nerve terminal plasma membrane and that both proteins are absent from synaptic vesicle membrane. However, proteins are differently distributed, the PMCA clusters are localized to the transmitter release sites while $Na^+$-$Ca^{2+}$-exchangers tend to be located at non-synaptic regions of the terminal (62).

The relative contribution of PMCA $Ca^{2+}$ pumps to the removal of $[Ca^{2+}]_i$ after a stimulus has led to some conflicting results. In a study on cerebellar Purkinje cell somata, SERCA pumps, PMCA pumps and $Na^+$-$Ca^{2+}$ exchangers accounted for most the $Ca^{2+}$ clearance at 500 nM $Ca^{2+}$. At 2 μM $Ca^{2+}$ the contribution of SERCA and the $Na^+$-$Ca^{2+}$-exchanger and especially PMCA pumps decreased, PMCAs accounting for only 6% (63). In contrast, in the study of Mironov (64) on hippocampal neurons, both PMCA and the organellar $Ca^{2+}$ pump were the main determinants for lowering cytosolic $Ca^{2+}$ under physiological conditions, whereas the $Na^+$-$Ca^{2+}$-exchanger and mitochondria seemed to play a minor role.

### 6.1. Experimental calcium overload

#### 6.1.1. Effect on PMCA expression

As has been described in section 4.1.1, PC-12 cells present an altered PMCA isoform expression pattern during culturing in $[Ca^{2+}]_i$ elevating conditions such as depolarization by high $K^+$. PMCA expression levels have been shown also to be critical to PC-12 cell survival in pathological situations such as $Ca^{2+}$overload and $Ca^{2+}$ mediated cell death (65). PC-12 cells overexpressing the PMCA4b isoform were less vulnerable to $Ca^{2+}$-mediated cell death induced by the $Ca^{2+}$ ionophore A23187 than control cells. Conversely, suppression of endogenous PMCA4 by the antisense method resulted in increased vulnerability to $Ca^{2+}$-mediated cell death. These data indicate that regulation of PMCA expression may be critical to cellular survival when cells are exposed to pathological increases in $[Ca^{2+}]_i$ and changes in $Ca^{2+}$ homeostasis.

#### 6.1.2. Effect on PMCA function as $Ca^{2+}$-$H^+$ exchanger

Changes of intracellular pH may have multiple effects on the regulation of cellular functions that may have significant pathological impact. The PMCA pump operates as an electrogenic $Ca^{2+}$-$H^+$ exchanger with a 1:1 stoichiometry (66). The increase of its activity during $Ca^{2+}$ signalling events may thus be involved in cellular acidosis. Such an effect has been clearly demonstrated in smooth-muscle cells (67). Since $Ca^{2+}$ and protons have opposite effects on many cellular processes, the role of PMCA in the regulation of these two ions may be of general importance.

As demonstrated on hippocampal neurons, brainstem slices and snail neurons, depolarization-induced $[Ca^{2+}]_i$ elevation evokes a prominent fall of intracellular pH ($pH_i$) caused by exchange of $Ca^{2+}$ for extracellular protons via a vanadate- and eosin-sensitive plasmalemmal $Ca^{2+}$-$H^+$ pump (68). A marked acidosis also occurs following glutamate stimulation of cerebral granule cells (69) and hippocampal neurons (70) with possible pathological consequences. Extracellular alkalinization decreases $Ca^{2+}$ extrusion by the inhibition of activity of PMCA pump-mediated $Ca^{2+}$-$H^+$ transport following a glutamate challenge. Comparison of the effects produced by alkaline pH and by the external $Na^+$ concentration led Khodorov and coworkers (70) to suggest that $Ca^{2+}$ pump mediated $Ca^{2+}$ extrusion rather than $Na^+$-$Ca^{2+}$-exchange may play a dominant role in the removal of $Ca^{2+}$ from glutamate-treated neurons. The physiological and pathological significance of $pH_i$ is further stressed by the observation that acidosis resulting from NMDA stimulation may constitute a negative feedback mechanism on the NMDA-activated ion influx (69). PMCA-induced proton transport thus contributes to the regulation of $pH_i$, in addition to $[Ca^{2+}]_i$ a potent component of neuronal excitability and synaptic plasticity.

### 6.2. Calcium-induced cell toxicity in *in vivo* conditions (pathologies)

#### 6.2.1. PMCA modifications in seizure and stroke

Prolonged seizures, complex partial seizures and status epilepticus may cause brain damage, resulting in death of susceptible cell types and fibrillary gliosis, most typically in hippocampus, amygdala and neocortex (71). Working on brain slices and using tetanic stimulation as a model for seizure, Pelletier *et al.* (71) concluded that $Ca^{2+}$ influx together with $Ca^{2+}$ release from endoplasmic $Ca^{2+}$ stores are necessary for seizure-induced cell death. The potential participation of PMCAs has been analyzed by studying the changing expression pattern in rat hippocampus during kainate-induced seizures (72). Relative mRNA levels for three PMCA isoforms (PMCA 1, 2 and 3) were examined by *in situ* hybridization. PMCA1 and PMCA2 were decreased in pyramidal cells of the CA1 and CA3 regions 12 hours following kainate injection. In some pyramidal cells, PMCA1 remained significantly below control levels for 72 hours post-injection. Decreased PMCA expression preceded the onset of neuronal loss. The distinct time course of the changes in PMCA1 and PMCA2 mRNAs and the lack of effect of kainate on PMCA3 expression suggests that early decreases in mRNAs can not simply be explained by cell death of pyramidal neurons. Interestingly, expression of PMCA2 showed a clear decrease in the granule cells of the dentate gyrus already 4 hours after injection. However, its expression quickly returned to control levels after 24 hours. It could be hypothesized that this observation is related to the fact that granule cells of the dentate gyrus are more resistant to excitotoxic and ischemic cell death (31, 73). Thus, the PMCA recovery seems to maintain $[Ca^{2+}]_i$ at levels more close to physiological during times that are crucial for cell survival.

In humans, the hippocampus expresses relatively high levels of PMCA2 and PMCA4 mRNAs. It could be speculated that the increased PMCA expression may contribute to the higher resistance of this region to excitotoxic injury.

A significant depression of $Ca^{2+}$-ATPase activity has also been observed on rat brain cortex following pentylentetrazol-induced status epileptic seizure and streptozotocin-induced diabetes (74). The observed decrease in $Ca^{2+}$-ATPase activity can reflect the diminution of the total number of PMCA protein molecules due to decreased translation or due to the decreased number of total cells, or could be due to a decreased number of active enzyme entities caused by fragmentation, aggregation or chemical alterations (49). The reduction of $Ca^{2+}$-ATPase activity following treatments inducing status epilepticus or diabetes may thus be interpreted as one of the initial biochemical lesions which start a cascade of processes which may later culminate in cell death.

Stroke and atherosclerosis in humans significantly affect erythrocyte membrane lipid composition and ATPase activities. Measurements on 51 patients with acute cerebral infarction and 12 patients with atherosclerosis revealed a decreased erythrocyte deformability, an increase in the membrane cholesterol/phospholipid ratio and a decrease of $Ca^{2+}$-ATPase and $Na^{+}$-$K^{+}$-ATPase activities. It is interesting that changes exist before the occurrence of stroke and could thus serve as an early indication of the patient's state in clinical settings (75).

#### 6.2.2. PMCA alterations in Alzheimer`s disease (AD)

The physical properties of cell membranes and calcium homeostasis in both the central and peripheral nervous system are changed in Alzheimer's disease. The amyloid beta-peptide (Abeta) that accumulates in the brain in AD is potentially neurotoxic. As shown by experiments on cultured hippocampal neurons, addition of Abeta causes loss of $Ca^{2+}$ homeostasis and neuronal injury which is preceded by impairment of the $Na^{+}$-$K^{+}$-ATPase activity (48). Although the authors consider the inhibition of $Na^{+}$-$K^{+}$-ATPase activity as the main cause of the disruption of $Ca^{2+}$ regulation, they also observe a reduction of $Ca^{2+}$-ATPase on exposure of synaptosomes prepared from postmortem human hippocampus to Abeta. Antioxidants such as vitamin E and propylgallate significantly protected cells against the ATPase impairments and neurotoxicity, suggesting a role for reactive oxygen (nitrogen species) in the pathogenesis caused by Abeta (48). A recombinant carboxy terminal 105 amino acid fragment of beta-amyloid precursor protein containing the full length of Abeta (CT 105) has *in vitro* a more potent effect than that of Abeta on cultured neuronal $Ca^{2+}$ uptake and $Na^{+}$-$Ca^{2+}$ exchanger (76). In addition, CT 105 disrupts calcium homeostasis and renders neuronal cells more vulnerable to glutamate-induced excitotoxicity in rat primary cortical neurons and PC 12 cells (77). It was not shown, however, that the effect directly involves PMCA activity.

Increased exposure to aluminium has been considered as one of the causative factors of AD. Mundy *et al.* (78) studied the effect of aluminium on the activities of $Ca^{2+}$ transport in microsomes and synaptosomes isolated from adult rat frontal cortex and cerebellum. In all organelles addition of 50-800 μM $AlCl_3$ resulted in a concentration-dependent inhibition of $Ca^{2+}$ transport. However, the same concentrations induced an activation of $Ca^{2+}$-dependent ATPase activities from both brain regions. The authors proposed that aluminium acts at multiple sites of neurons to displace both $Ca^{2+}$ and $Mg^{2+}$ thereby increasing the ATPase activity but disrupting transport of $Ca^{2+}$. It has, however, not been proven that any of these effects involve PMCA activity rather than organellar $Ca^{2+}$ pumps.

The hypothesis that abnormalities in cellular $Ca^{2+}$ regulation might be involved in the etiology of AD was further supported by experiments on fibroblasts from AD patients (79). The $Mg^{2+}$-dependent, $Ca^{2+}$-

stimulated ATPase presented an approximately two fold higher $K_m$ value in Alzheimer fibroblasts than in controls. Although the authors ascribe this difference to an effect on PMCA, this interpretation awaits further proof because the experiments were performed on crude homogenates.

### 6.2.3. Structural and functional alterations of PMCA in brain hypoxia/ischemia

Although the $Ca^{2+}$-dependent processes seem to play a major role in cell damage, the timing of $Ca^{2+}$-dependent damage and the role of increased ability to buffer or reduce cytoplasmic $Ca^{2+}$ loads in ischemic tolerance are still unresolved. Ischemic death of specific neurons shares some of the mechanisms of classical forms of apoptosis and necrosis. Studies on lower mammals have suggested that neuronal death after brief ischemia in the CA1 hippocampal region occurs by apoptosis. However, studies on monkeys and humans rather support necrosis, the calpain-cathepsin dependent cell degeneration, as the primary mechanism (80).

In Jurkat cells and neuroblastoma cells undergoing apoptosis, only $IP_3$ receptor type 1 has recently been shown as a caspase 3 substrate among the proteins associated with $Ca^{2+}$transport (81, 82). Digestion of $IP_3$ receptors may interfere with $Ca^{2+}$-signaling pathways and intracellular $Ca^{2+}$ homeostasis. These results provide an explanation for the lower levels of this protein in AD and other neurodegenerative diseases (82).

On the other hand, ion transport systems may also be decisive to cell death. It has been recently shown by (83) that caspases can cleave different plasma membrane $Ca^{2+}$ pump isoforms and thereby cause membrane lysis in cells undergoing apoptosis.

In recent work from our own laboratory we have attempted to investigate the potential role of PMCAs in altered $Ca^{2+}$ homeostasis following transient global ischemia and prolonged reperfusion. Isoform-specific antibodies against three human isoforms of PMCA but recognizing the corresponding protein in the rat raised by Stauffer (84, 85) were used for immuno-localization of PMCAs in the different regions of gerbil brain. Similarly to human and rat tissues, all three gene products (PMCA1, 2 and 3) were detected in the hippocampus, frontal cerebral cortex and cerebellum. PMCA1 was expressed in the highest quantity in the cortex and hippocampus. The PMCA2 signal was generally lower than that of PMCA1 and was highest in the cerebellum, with a moderate amount in the cortex. Anti-PMCA3 antibody stained weakly and was localized in the cerebellum and in the hippocampus. As expected from our previous results using non-isoform discriminating antibodies (86), 10 min global forebrain ischemia had no effect on the level of any PMCA isoforms. However, ischemia followed by reperfusion led after up to 7 to 10 days to a significant decrease of the PMCA immuno-signal in the forebrain membranes. This decrease could be ascribed solely to the selective loss of PMCA1 signal (to 59 and 63 % of control levels) and the major loss was localized to the hippocampus, which is thought to be the most affected brain region (CA1 sector) after global ischemia. PMCA2 and PMCA3 did not show significant differences after IRI neither in the hippocampus nor in the cerebral cortex. Combined with the observation that the tubulin level does not differ significantly between control and ischemic tissue, these data demonstrate that the decrease of PMCA1 can not simply be explained by cell death or a general response of all isoforms to ischemia/reperfusion injury (87). We don't know yet whether the major effect on PMCA1 level occurs at the stage of transcription, translation or degradation.

A possible explanation arises from the experiments of Neumar´s group (88, 89). Transient ischemia in rats induced by bilateral carotid occlusion and hypovolemic hypotension followed by 48 hours reperfusion induced proteolytic fragmentation of PMCA protein in parallel with a considerable depression of $Ca^{2+}$-ATPase activity to 51 per cent prior to morphological evidence of neuronal degeneration. Proteolysis in the hippocampus was caused by increased calpain activity. However, the antibodies used in this study (5F10) did not discriminate between individual isoforms and thus direct evidence concerning the selective loss of PMCA1 in gerbil hippocampus is still lacking. Whether PMCA1 is more than other isoforms vulnerable to excitotoxic injury or to action of free radicals (protein degradation is under redox control) (55), in ischemia is not yet known. However its selective decrease in the most affected area suggests a specific role in the dynamic regulation of $[Ca^{2+}]_i$ and in the derangement of $Ca^{2+}$ homeostasis leading to the delayed death of hippocampal neurons. It should be remarked that in parallel with the decrease of PMCA a number of other $Ca^{2+}$ transport proteins are affected during the reperfusion period: the intracellular $Ca^{2+}$ pump (90, 91) and the $IP_3$ receptor (92).

Although a similar progressive reduction of histochemically determined $Ca^{2+}$-ATPase activity has been documented in gerbil CA1 hippocampal neurons during reperfusion period (93), the results are less indicative due to the unspecificity of the histochemical determination. However, severe disturbances of the $Ca^{2+}$-ATPase precede the early stages of delayed neuronal death and thus seem to occur far earlier than the morphological degenerative changes or increased calcium content in the CA1 pyramidal cells.

Interestingly, the ubiquitin/proteasome pathway is thought to play an important role in cachexia, characterized by profound tissue wasting, that frequently complicates malignancies. The status of the $Ca^{2+}$-dependent proteolytic system has been evaluated in the gastrocnemius muscle of AH-130 tumor bearing rats

by measurement of the activity of calpain, of calpastatin, its natural inhibitor, and of the PMCA protein, a calpain substrate. After tumor transplantation, a decrease in PMCA $Ca^{2+}$-ATPase was the prominent feature, while the level of the SERCA pump did not change. The diminution of PMCA protein is caused by the progressively increasing dysbalance in the calpain/calpastatin ratio since the level of total calpastatin activity progressively declined, while total calpain activity remained unchanged. Moreover, decreased levels of both calpastatin and plasma membrane $Ca^{2+}$-ATPase have also been detected in the heart of the tumor-bearers, which strongly suggests a more general $Ca^{2+}$-dependent proteolysis which may play a role in the muscle protein hypercatabolic response (94).

A clinically very interesting situation is the pretreatment by non-lethal ischemic loading in CA1 hippocampal neurons (95), which is thought to cause protection against $Ca^{2+}$ toxicity (ischemic tolerance). As shown in ischemia-tolerant gerbils, the level of $Ca^{2+}$-ATPase determined by histochemical methodology in CA1 neurons exposed to a non-lethal 2 min ischemia, increased significantly before the start of the 5 min of lethal ischemia and even remained at a higher level subsequently, compared to ischemia non-tolerant CA1 neurons. This result strongly resembles data on CA3 neurons, which are constitutively resistant to such periods of ischemia (96). However, due to the methodology used in $Ca^{2+}$-ATPase analysis and because of a parallel enhanced mitochondrial $Ca^{2+}$ buffering system in tolerant regions, the role of $Ca^{2+}$-ATPase in the process of acquired tolerance to subsequent ischemia might be questioned.

In fact, we recently showed that the extent of lipid and protein modifications in a whole rat neuronal preparation induced by IRI occurs mainly during the period of reperfusion. Similarly, an increase of immunoreactivity for HNE-modified proteins in CA1 hippocampal region was seen in the reperfusion period after transient ischemia in gerbils. Loss of immunoreactivity increase in ischemic tolerant animals points to the important role of HNE in oxidative stress-induced altered neuronal signalling and survival of neurons (54).

Chronic cerebral vasospasm as a consequence of subarachnoid hemorrhage (SAH) has also been shown to be associated with impaired $Ca^{2+}$ regulation in smooth-muscles of cerebral arteries (97). Experimental SAH in the canine basilar artery caused a decrease of the activity of plasma membrane $Ca^{2+}$-ATPase as a result of single or double experimental blood injection. Concomitantly there was an increase of the plasma membrane permeability to $Ca^{2+}$. The harmful substances in the extravasated blood and other factors such as superoxide which are released from the oxyhemoglobin in a subarachnoid clot might be causative agents in the early occurrence and long lasting decrease in $Ca^{2+}$-ATPase in experimental SAH.

### 6.3. Role of PMCA in myocardial pathologies

In cardiomyocytes the pathway of $Ca^{2+}$ efflux via the $Na^{+}$-$Ca^{2+}$ exchanger has a higher maximal capacity than that via the $Ca^{2+}$-transporting ATPase. However, the sarcolemmal $Ca^{2+}$ pump becomes important at the lower $[Ca^{2+}]_i$ during diastole due to its higher affinity for $Ca^{2+}$ (98, 99).

Cardiac dysfunction following ischemia is accompanied by a general imbalance of ion transport, particularly of $Ca^{2+}$. The effect of cardiac ischemia on ATP-dependent $Ca^{2+}$ transport of sarcolemma has been investigated in several laboratories, with confirming but also some conflicting results.

In an early study, Chemnitius *et al.* (100) have investigated the properties of sarcolemmal membranes purified from isolated perfused canine hearts following intermittent clamping of the inflow and outflow until a stable decline in the rate of force development had been reached. This treatment resulted in irreversible damage to ATP-dependent sarcolemmal $Ca^{2+}$ transport, with depression of both the initial rate of $Ca^{2+}$ uptake and maximal $Ca^{2+}$ uptake capacity. Dhalla and coworkers have observed similar effects and have moreover shown that reoxygenation induces a further decline of ATP-dependent $Ca^{2+}$ transport of the sarcolemma (101, 102). $Ca^{2+}$ pumping activity declined with the duration of ischemia and was accompanied by structural changes of the sarcolemma (103).

Samouilidou *et al.* (104) have observed a depression of the $Ca^{2+}$-stimulated ATPase activity of sarcolemmal membranes of ischemic hearts *in situ*. As a model, dogs subjected to systemic hemodialysis were used, with 90-min ligation of the coronary artery followed by 30-min reperfusion. The inhibition of sarcolemmal ATP-dependent $Ca^{2+}$ transport was reflected in a decrease of the $V_{max}$ and an increase of $K_m$ for calmodulin. Interestingly, reduction of the $Ca^{2+}$ concentration had a protective effect on the ischemia-induced alterations of the calmodulin-stimulated $Ca^{2+}$-ATPase activity. The authors propose that the decrease of $Ca^{2+}$-ATPase activity after ischemia is caused by alterations of the calmodulin-binding site of the enzyme.

An insensitivity of PMCA activity to ischemia was found by (105). In sarcolemmal vesicles from rabbit heart, the initial velocity of ATP-dependent $Ca^{2+}$ transport was unaffected even by 2 hours of ischemia, whereas the $Na^{+}$-$Ca^{2+}$-exchange activity was significantly inhibited and the number of $Ca^{2+}$ channels was reduced. The maintenance of normal function of the ATP-dependent $Ca^{2+}$ pump indicates that there is not simply a gross disruption of all sarcolemmal function during ischemia in this model.

Oxidative stress may play a crucial role in cardiac dysfunction, including ischemia/reperfusion injury (for review, see 106). Dhalla and coworkers have investigated the effect of oxidative stress on PMCA function in a series of studies, both on perfused hearts and directly on isolated sarcolemmal membranes. Depression of sarcolemmal PMCA activity similar to that in ischemia/reperfusion was observed by cardiac perfusion with radical-generating solutions. In ischemia as well as with radical-generating perfusion, scavenging enzymes prevented the effects. The ATP-dependent $Ca^{2+}$ transport of purified membranes from control hearts was inhibited by oxygen radicals, and also this effect was reversed by antioxidant enzymes (98, 107). Similar effects have also been observed on coronary artery smooth-muscle cells (108).

Cardiac hypertrophy and myopathy are other conditions associated with abnormalities in $Ca^{2+}$ handling, including modifications of the ATP-dependent $Ca^{2+}$ transport in the sarcolemma. Sarcolemmal membranes from both pressure-induced (aortic banding for 28 days) and hypertension cardiac hypertrophy in rats present an increased activity of the sarcolemmal $Ca^{2+}$ pump in parallel with altered phospholipid composition and number of voltage-dependent $Ca^{2+}$ channels. The results suggest that alterations in the sarcolemmal $Ca^{2+}$ transport activities may serve as an adaptive mechanism facilitating the removal of $Ca^{2+}$ from the myocardial cells during the development of cardiac hypertrophy (109). On the other hand, in the development of clinical symptoms of hereditary cardiomyopathy in hamsters, the sarcolemmal $Ca^{2+}$-ATPase is decreased in parallel with lesion development. Both the $K_m$ for $Ca^{2+}$ and the $V_{max}$ are affected. Because the activities of $Na^+$-$K^+$-ATPase and $Na^+$-$Ca^{2+}$-exchanger were unaltered, and the number of $Ca^{2+}$ channel receptor binding sites was increased, an imbalance in $Ca^{2+}$ fluxes appears to be involved in the pathogenesis of the disease, with an important contribution of altered PMCA function (110). In addition, certain cardiomyopathic side effects of drug administration may be explained by free-radical induced inhibition of ATP driven $Ca^{2+}$ transport.

ROS possibly in the form of hydrogen peroxide generated by the metabolism of alloxan inhibit $Ca^{2+}$ transport by plasma membranes isolated from dog aorta. This action may contribute to the vasoconstrictive side effect of this drug (111).

The role of oxidative stress may not be restricted to ischemia/reperfusion-related dysfunction. Oxidative damage may also be an important factor in cardiomyopathy caused by excessive amounts of circulating catecholamines. Experimental treatment of rats with a high dose of the synthetic catecholamine isoproterenol resulted, in addition to morphological and functional changes, in an increased level of oxidative stress products such as malondialdehyde content, conjugated dienes and low glutathione redox ratio. In parallel, depression of cardiac sarcolemmal ATP-dependent $Ca^{2+}$ uptake, $Ca^{2+}$-stimulated ATPase activity, and $Na^+$-dependent $Ca^{2+}$ accumulation was observed. Similar results have been obtained in isolated hearts perfused with adrenochrome (an oxidized product of catecholamines). These effects were ameliorated by vitamin E. Thus, intracellular $Ca^{2+}$ overload partially caused by defects in sarcolemmal transport, may play an important role in catecholamine-induced cardiomyopathy (112).

Data on expression or function of the sarcolemmal calcium pump in heart failure are not available (113).

## 7. THE ROLE OF PMCA IN OTHER TISSUE PATHOLOGIES

### 7.1. The role of PMCA in liver pathologies

In the liver, as in other organs, many cellular processes are activated by increased levels of $[Ca^{2+}]_i$. Amongst others, gluconeogenesis, cellular respiration, glycogenolysis and cell division are examples of $Ca^{2+}$-dependent processes (114). Properties of the liver plasma membrane $Ca^{2+}$-transport ATPase are different from those of other cell types. The liver $Ca^{2+}$ pump is not stimulated by calmodulin. It possesses a high affinity for $Ca^{2+}$ in the absence of modulators and it is activated by a specific protein, regucalcin (115). The enzyme is located predominantly in the blood sinusoidal endothelial fenestrae domain of the hepatocytes.

Regucalcin is a $Ca^{2+}$-binding protein that does not contain an EF-hand motif. Regucalcin plays an important role as a regulatory protein in $Ca^{2+}$ signalling by activation of the PMCA pump in the basolateral membranes of liver and in renal cortex cells (115). Hepatic regucalcin mRNA expression in rats has been shown to be stimulated by various factors including $Ca^{2+}$, calcitonin, insulin, and estrogen. The mRNA is also expressed in hepatoma cells.

The cytoskeleton and tight junctions are important for maintenance of the polar distribution of plasma membrane proteins in bile canicular membranes of hepatocytes. As a response to cholestasis, a redistribution of a $Ca^{2+}$-ATPase activity in hepatocytes of rat liver has been reported. However, this enzyme activity has recently been identified as an ecto $Ca^{2+}$-ATPase (116, 117).

Mas Oliva *et al.* (118) compared the $Ca^{2+}$-ATPase and $Ca^{2+}$-transport activity of plasma membrane fractions from normal liver, regenerating liver and from AS-30D ascites hepatocarcinoma cells. The regenerating liver and the AS-30D plasma membrane fractions presented a much lower ATPase activity than the corresponding fractions from normal liver, whereas the $Ca^{2+}$-transport activity was similar. The authors propose

a more efficient mechanism to regulate the movement of $Ca^{2+}$ through the $Ca^{2+}$ pump in hepatocarcinoma cells.

The accumulation of intracellular calcium caused by ischemia/reperfusion during liver transplantation has been implicated as a factor leading to primary graft nonfunction. Porcine liver, cold-preserved on ice, did not significantly differ in $Ca^{2+}$-ATPase activity (0.47 nmol/mg/min) from human liver. Because $Ca^{2+}$-ATPase from rat liver was significantly higher, porcine liver may be a better model for studies of human liver. Cold-preservation experiments on porcine liver for clinically relevant periods showed a considerable decrease of plasmalemmal $Ca^{2+}$-ATPase activity after 4 hours storage and near-complete inhibition after 12 hours, which was more pronounced than that of SERCA $Ca^{2+}$-ATPase. This supports the hypothesis that preservation ischemia initiates time-dependent calcium accumulation with potential consequences for altered $Ca^{2+}$ homeostasis (119).

The cellular dysfunction that eventually leads to multiple organ failure in sepsis is unclear. Sepsis is characterized by impaired hepatic glucose homeostasis and muscle protein metabolism, all processes that involve $Ca^{2+}$. As shown by (120), the impairment of the capacity to extrude $Ca^{2+}$ from erythrocytes as well as from liver plasma membranes appears to have a general pathophysiological significance in the development of severe sepsis. The degree of inhibition of the $Ca^{2+}$ pump depends on the severity of the septic process, indicating that $Ca^{2+}$ pump activity represents a potentially useful marker for general cellular dysfunction to evaluate the clinical manifestations of sepsis.

### 7.2. Functional alterations of PMCA in diabetes

The $Ca^{2+}$ ion is deeply involved in the regulation of insulin secretion. Secretagogues induce a significant increase of $[Ca^{2+}]_i$ in beta cells which is dependent on extracellular $Ca^{2+}$. Depending on the extracellular level of glucose, $[Ca^{2+}]_i$ in beta cells undergoes oscillations whose duration depends on the extracellular glucose concentration (121). Beta cells and other pancreatic islet cells of pancreas contain a unique combination of PMCA pump protein isoforms, predominantly PMCA1b, 2b, 3a, 3c and 4a. Thus, many functionally distinct PMCA isoforms participate in $Ca^{2+}$ homeostasis in insulin secreting cells (122, 123). It appears that cyclic $Ca^{2+}$ entry from the extracellular space in parallel with modulation of PMCA activity and $Ca^{2+}$ release from the ER contribute to the glucose-induced cytosolic $Ca^{2+}$ rise (124).

The plasmalemmal $Ca^{2+}$-ATPase activity of pancreatic islet cells is suppressed by high glucose, as can be concluded from different experimental approaches. The ATP- and $Mg^{2+}$-dependent $Ca^{2+}$ uptake of purified plasma membranes is inhibited for 50% by 30 mM glucose (125). Levy *et al.* (126) incubated pancreatic islets *in vitro* in the presence of different glucose concentrations. Increasing concentrations caused a progressive inhibition of the $Ca^{2+}$-stimulated ATPase activity of plasma membranes isolated from these cells. In the same line, islets isolated from non-insulin-dependent diabetes mellitus rats had lower $Ca^{2+}$-ATPase activity than normal controls (126). Thus, the glucose blindness of the diabetic islet may be explained as a result of defective cellular $Ca^{2+}$ metabolism, and decreased $Ca^{2+}$-ATPase activity may contribute to this pathology.

Cumulative evidence reveals that diabetes is a condition in which cell $Ca^{2+}$ homeostasis is impaired in many cell types, confirming that this impairment is a basic phenomenon associated with diabetic status (127). Defects in cell $Ca^{2+}$ regulation were found in erythrocytes, cardiac muscle, platelets, liver, skeletal muscle, kidney, aorta, adipocytes, osteoblasts, peripheral nerves, retinal tissue, brain and beta pancreatic cells. Although an increase in $[Ca^{2+}]_i$ is the most common finding, it is not always clear whether the defect in cell $Ca^{2+}$ precedes or succeeds the diabetic condition. It is also not clear which of the transport systems for $Ca^{2+}$ is the primary defect. As in pancreatic islets, alterations in $Ca^{2+}$-ATPase or $Ca^{2+}$-transport activities have been documented in plasma membranes from various tissues, both in experimentally induced diabetes and in human patients with type 1 or type 2 diabetes (128, 129). Some of the changes are, however, contradictory.

Erythrocytes are the most commonly studied cell type, probably because erythrocyte membranes are the most suitable system to investigate plasmalemmal active $Ca^{2+}$ transport. Several authors have shown that erythrocytes of diabetic patients present an increased level of intracellular $Ca^{2+}$ and diminished $Ca^{2+}$-ATPase activity in comparison to healthy individuals (130, 131). The activity depends on the clinical status, e.g. poorly controlled diabetic patients (132). The altered PMCA activity may indirectly contribute to the development of chronic complications of diabetes. In addition, in type 2 diabetes but not in type 1 a significant correlation between the decrease of $Ca^{2+}$-ATPase and creatinine clearance was shown, suggesting that this activity decrease may predispose to the development of hypertension in diabetes mellitus. In human diabetes type 1, sex and parental hypertension are predictor factors correlated with a greater depression of erythrocyte $Ca^{2+}$-ATPase activity and which may indirectly be linked to increased susceptibility towards vascular complication (133).

Studying erythrocytes in type 2 diabetic patients, Migdalis *et al.* (134) observed abnormalities of erythrocyte $Ca^{2+}$-ATPase only in those patients with peripheral neuropathy. Because erythrocyte $[Ca^{2+}]_i$ is an important regulator of erythrocyte deformability, the altered PMCA activity and associated microangiopathy is thought to participate in diabetes-induced pathogenesis of neuropathy. In another study, Muzulu *et*

*al.* (135) observed that both basal and calmodulin-stimulated $Ca^{2+}$ pump activities of erythrocytes were reduced in diabetic patients. Because there was no evidence for modifications of the membrane lipids, a direct effect was proposed of the diabetic condition on the $Ca^{2+}$-pump protein itself. These results correlate with findings of (136) and (137) who demonstrated that *in vitro* glycation of the $Ca^{2+}$ pump diminishes $Ca^{2+}$-ATPase activity by up to 50%. This decrease was explained by the reaction of glucose with one essential Lys residue probably located in the vicinity of the catalytic site. The higher glucose concentration inhibits in a time-dependent manner the activity of purified $Ca^{2+}$-ATPase reactivated by defined phospholipids, suggesting a direct glycation of the enzyme (137). This interpretation has been questioned, however, by more recent findings. Normal human red blood cells exposed to high glucose concentrations (30 -100 mM) for 6 hours *in vitro* did not exhibit any effect on $V_{max}$ of PMCA. Also the mean $V_{max}$ from diabetic subjects was not significantly different from that of controls. Results on experimental diabetes induced by streptozotocin in rats were in the same line. These studies question the previous findings and support the notion that a high glucose concentration in plasma is not a causative factor for the altered ability of red blood cells to extrude $Ca^{2+}$ (138).

Lipoic acid lowered lipid peroxidation and protein glycosylation, and increased $Na^{+}$-$K^{+}$- and $Ca^{2+}$-ATPase activities in high-glucose exposed red blood cells. This effect could be the mechanism by which lipoic acid may delay or inhibit the development of neuropathy in diabetes and may form the basis of a new therapeutic approach (139).

Lymphocytes are also affected by diabetes. Basal $[Ca^{2+}]_i$ is elevated in diabetic type 2 subjects and is positively correlated with the glucose and hemoglobin A1c level. In addition, a significant reduction of PMCA $Ca^{2+}$-ATPase activity was detected in diabetic subjects compared to controls which suggests that $Ca^{2+}$ accumulation in the cell is at least partially caused by depression of PMCA activity (140).

In renal cortical cells, Levy and Gavin (141) have shown not only a diabetes-induced decrease of plasma membrane $Ca^{2+}$-ATPase activity but also a direct regulation of this activity by phosphorylation of the enzyme induced by insulin. They further demonstrated in genetically obese rats that loss of insulin regulation of ATPase activity may impair insulin effects and therefore contribute to insulin resistance. Contrary to this report, Evcimen *et al.* (142) observed an elevation in $Ca^{2+}$-ATPase activity on whole kidney in streptozotocin-induced diabetes. This increase may have been caused by higher $Ca^{2+}$ levels in diabetic kidneys resulting from a compensatory response of the enzyme to high levels of the ion.

Brain synaptic PMCA activity is inhibited by chronic hyperglycemia. The inhibition is also associated with a significant reduction of the minimum effective dose for inhalation anesthetics in experimental animals (143). Because inhalation anesthetics themselves also cause an inhibition of PMCA activity, the authors suggest that in specific brain regions PMCA could play an important role in inhalation anesthesia. Oner *et al.* (74) confirmed the diabetes-induced decrease of brain $Ca^{2+}$-ATPase activity and in addition observed such an effect as a consequence of experimentally induced seizures.

Oxidized low density lipoproteins (LDL) probably play a pathogenic role in diabetes-induced and non-diabetes-induced atherosclerosis by damaging both endothelial barriers and blood-cell membranes. In platelets, oxidized LDL but not native LDL (in concentrations considerably below the level of circulating LDL) increases the cytoplasmic $Ca^{2+}$ content and inhibits the activity of the $Ca^{2+}$-ATPase of the plasma membrane. Since no change in membrane fluidity was observed in platelets exposed to oxidized LDL, a direct inhibition of PMCA protein has been proposed. In clinical settings such as diabetes and hypertension, altered $Ca^{2+}$-ATPase activity in platelets might contribute to the increase of $[Ca^{2+}]_i$ (40). LDL from normolipidemic insulin-dependent diabetic patients significantly increased the *in vitro* platelet aggregation response to ADP compared with platelets incubated with control LDL (144). An altered interaction between circulating lipoproteins and platelets is thus suggested as a new mechanism of the early development of atherosclerosis in diabetic patients.

In contrast to the changes induced in most other tissues, the ATP-dependent $Ca^{2+}$ uptake and $Ca^{2+}$-ATPase activity in sarcolemmal fractions from skeletal muscle appear to be increased in diabetic animals (145). These changes were apparent three or four weeks after streptozotocin administration. Treatment of diabetic animals with insulin for 14 days reversed the changes in $Ca^{2+}$ transport activities toward the control levels. On the other hand, sarcolemmal $Mg^{2+}$-ATPase and $Na^{+}$-$K^{+}$-ATPase activities remained unchanged and no difference in the sarcolemmal phospholipid composition and protein electrophoretic pattern was evident between the control and experimental groups. The higher activity of the sarcolemmal $Ca^{2+}$ transport is probably associated with hyperfunction of skeletal muscle in diabetic rats. However, it appears that in cardiac muscle, like in most cell types, the sarcolemmal $Ca^{2+}$ pump is defective in diabetic myocytes (146).

### 7.3. Abnormalities of PMCA in erythrocyte-related pathologies

Abnormalities of the $Ca^{2+}$ pump of erythrocytes, possibly reflecting systemic alterations of the pump in other tissues as well, have been described in several disease conditions (147).

In sickle cell anemia, circulating red blood cells are heterogeneous in age, morphology, volume, membrane structure and ion transport. Much of this heterogeneity is the result of the polymerization of hemoglobin which leads to secondary abnormalities of cell ion and water content. Sickle red cells (SS cells) have an abnormally high total $Ca^{2+}$ content which increases with reticulocyte maturation.

The oxidation of hemoglobin to methemoglobin and its degradation leads to the accumulation of hemin and free iron in the red blood cells. The $Ca^{2+}$-ATPase activity of erythrocytes has been shown to be inhibited by different types of hemolytic amenia such as sickle cell disease or glucose-6-phosphate dehydrogenase deficiency. Leclerc *et al*. (33) have shown that the erythrocyte $Ca^{2+}$-ATPase activity is inhibited by hemin, the ferric protoporphyrin IX (FP) complex. Because the inhibition of the enzyme was partially restored by the reducing agent dithiotreitol, it was concluded that hemin induces the oxidation of thiol groups of the protein. The next Leclerc study (148) demonstrated that non-heme iron also inhibits the calmodulin stimulation of the $Ca^{2+}$-ATPase activity. In contrast with hemin, the non-heme iron inhibition is prevented by butylated hydroxytoluen, a protective agent of lipoperoxidation, and dithiotreitol does not restore the enzyme activity.

High levels of membrane-bound ferriprotoporphyrin IX (FP) and non-heme iron have been found in abnormal red cells such as SS cells and malaria-infected red cells, associated with a reduced life span. Although previous *in vitro* studies have shown that FP and non-heme iron have a marked inhibitory effect on the $Ca^{2+}$-ATPase activity of isolated red cell membranes (33, 148), Tiffert *et al.* (149) have shown that in intact SS cells, FP and non-heme iron are unlikely to play a significant role in the altered $Ca^{2+}$ homeostasis of abnormal red cells. On the other hand, a new route via activation of protein kinase C alpha (PKC) directly stimulating the $Ca^{2+}$ pump and preventing cell dehydration has been proposed in SS cells (150). A higher level of PKC alpha and PKC zeta expressed in SS cells directly stimulates the $Ca^{2+}$ pump and inhibits deoxygenation-induced $K^+$ efflux in SS cells.

Romero and Romero (151) point to difficulties with studies in an animal model of sickle anemia. Transgenic mice that express high levels of human alpha H and beta S-chains had a relatively small percentage of dense cells and do not exhibit anemia. Although the cells have a similar $Ca^{2+}$-activated $K^+$ channel activity to human SS cells, they express a very high $Ca^{2+}$-pump activity. These properties may contribute to the smaller percentage of very dense cells and to the lack of adult anemia in this animal model.

Abnormalities in erythrocyte $Ca^{2+}$ homeostasis are associated also with other pathologies. Familial phosphofructokinase deficiency is linked with an increased energy demand by the $Ca^{2+}$ pump to compensate the dissipating $Ca^{2+}$ gradient across the plasma membrane in human erythrocytes. The disease is associated with disturbed $Ca^{2+}$ homeostasis, alterations of the intracellular volume of red blood cells, and activation of the Gardos effect. The concomitant hemolysis may be explained by a diminished erythrocyte deformability due to $Ca^{2+}$ overload (152).

Erythrocyte membranes in protein-energy malnutrition (kwashiorkor- KWA) which is clinically linked with anemia contain a detectable amount of non-heme iron which causes lipid peroxidation and impairment of membrane functions. In addition, isolated KWA membranes are more susceptible to oxidative stress which is expressed in a lower affinity of the $Ca^{2+}$-ATPase for ATP and calmodulin. The excessive generation of ROS and the higher sensitivity of the membranes to oxidative stress may be responsible for the altered red blood cell $Ca^{2+}$ homeostasis in this pathology (153).

Tarui´s disease is another pathology with increased $Ca^{2+}$ content of erythrocytes. Due to the enhanced erythrocyte $Ca^{2+}$ permeability in all patients, compensatory mechanisms such as increased $Ca^{2+}$ pump activity are proposed to be initiated (154).

Clinically very interesting is the toxic effect of chronic exposure to low lead concentrations in the environment. Human patients with lead toxicity develop hematological, gastrointestinal and neurological dysfunction. In 247 mothers and their newborns, maternal and cord blood has been sampled at delivery. Erythrocyte $Ca^{2+}$-ATPase activity was correlated with the lead content in the blood. The results have shown that the level of lead in maternal and cord blood and mothers‘ hair was negatively correlated with the red blood cell $Ca^{2+}$-ATPase activity, suggesting that lead toxicity can at least in part be explained by the inhibition of the red blood cell $Ca^{2+}$-ATPase (155).

An abnormal red-cell $Ca^{2+}$ pump has also been found in idiopathic hypercalciuria, a common disorder with possible enzyme abnormalities in $Ca^{2+}$ transport. Erythrocytes in patients with hypercalciuria and history of calcium oxalate kidney stones expressed an increased $Ca^{2+}$-ATPase activity compared to healthy controls. Interestingly, urinary calcium excretion correlated well with the $Ca^{2+}$-ATPase activity, suggesting a relation between $Ca^{2+}$-ATPase activity and hypercalciuria (156). However, in a more recent study (157), a group of twenty-four children with idiopathic hypercalciuria did not show any changes in the abundance of erythrocyte PMCA protein or in $Ca^{2+}$-ATPase activity compared to control children.

Friedrichs and Wurz (158) have pointed to an interesting aspect of the erythrocytes of children

patients. The calmodulin-stimulated red-cell $Ca^{2+}$-pump activity was higher in females than in males. There were no differences in basal ATPase activity. The basal activity of PMCA in children up to the age of 7 years was higher than that of adults. A distinct increase in activation by calmodulin was observed with a maximum at the age range of 8-13 years. These data should be taken into account for the setting of normal values of the population.

An alteration in the $Ca^{2+}$ pump in red blood cells (higher $V_{max}$, lower $K_m$) has been described in patients with Duchenne muscular dystrophy (159). The change was specific for Duchenne dystrophy because it did not occur in other forms of dystrophy.

In patients with bipolar illness there is a much greater scatter in the $Ca^{2+}$ dependence and calmodulin sensitivity of the $Ca^{2+}$ pump than in normal subjects (160). Meltzer *et al.* (161) reported that the activity of erythrocyte calmodulin-stimulated $Ca^{2+}$-ATPase is higher in lithium-treated bipolar subjects than in normal controls, or than in bipolar subjects treated with psychotropic medications without lithium. This difference was due an increase of the number of $Ca^{2+}$ pumps.

Analysis of $Ca^{2+}$-ATPase activity of red-cell membranes from patients with cystic fibrosis revealed a reduced activity (162). A lower $V_{max}$ of ATP hydrolysis was also observed by (163). However, it can be questioned whether these effects occur specifically on the PMCA pump because of the general disturbance of the membrane lipid environment in this pathology.

Young and old human red blood cells, separated on Percoll gradients have been compared with respect to osmotically-induced $Ca^{2+}$ entry and $Ca^{2+}$ extrusion. The selective blockade of $Ca^{2+}$ influx by $Gd^{3+}$ suggests the presence of mechano-sensitive channels, that become preferentially activated in old cells and may explain the increased $Ca^{2+}$ content of senescent cells (164). In addition, either at pH 7.0 or 7.4, the maximal $Ca^{2+}$ extrusion rate (PMCA activity) in older (dense) cells was only one-half of that in younger cells (164). The results demonstrate that the elevated $[Ca^{2+}]_i$ in senescent cells, arises in part from a reduction in $Ca^{2+}$ extrusion (PMCA) capacity during aging. Xanthine derivatives affect erythrocyte deformability and $Ca^{2+}$ entry pathways and are used in the treatment of peripheral vascular disorders seen in elderly people and several pathologies. Pentoxyphilline (0.5 -5 mM), a dimethylxanthine, added directly to the assay medium activates the $Ca^{2+}$-ATPase of inside-out red-blood cell vesicles of young and old patients. However the activation was age-related with the greater percentage of activation in vesicles from erythrocytes of older patients. Pentoxyphilline also affected $Ca^{2+}$ uptake activity. The early burst of $Ca^{2+}$ uptake decreased in the vesicles from young but not from older erythrocytes (165).

### 7.4. Plasma membrane $Ca^{2+}$ pump in kidney disorders

In the mammalian nephron, 95 percent or more of the $Ca^{2+}$ filtered in glomeruli is reabsorbed in tubular segments. Although a substantial portion of the tubular reabsorption, especially in the proximal part, is a passive process, there is a considerable contribution of PMCA pumps to the overall absorption. The $Ca^{2+}$-ATPase activity overlaps with the cellular localization of mRNAs encoding PMCA proteins. PMCA1 is a house-keeping form, mostly present in non-epithelial interstitial cells of glomeruli. PMCA2 is detected in epithelial cells of cortical tubules. PMCA3 is located mainly in the outer medulla (166) and thin descending loop of Henle (167). PMCA4 is present in the outer medullary zone. In kidney tubular cells, as in intestinal epithelia, calcitriol and vitamin $D_3$ upregulate the expression and the activity of the 1b isoform of the plasma membrane $Ca^{2+}$ pump (168). In MDCK (Madin-Darby canine kidney) cells, a model of distal tubular cells, it has recently been shown for the first time that $Ca^{2+}$ inhibits its own transcellular transport. This inhibition is mediated by a $Ca^{2+}$-sensing receptor which inhibits in a concentration-dependent manner PMCA activity, via phospholipase C-dependent pathways (169).

The expression of PMCA isoforms can be selectively modulated by pathophysiological stimuli. Unlike the constant expression level of PMCA1 and PMCA4 isotypes, the expression of PMCA2 and PMCA3 in tubules has been shown to be depressed in hypercalciuric rats (induced by feeding with a low-phosphate diet) compared to hypocalciuric rats. In brain and liver, however, PMCA2 and PMCA3 were unchanged, suggesting a specific role for these isoforms in tubular $Ca^{2+}$ reabsorption (170).

Familial benign hypercalcemia (FBH) has been associated with defects in parathyroid-gland regulation, intrinsic renal hyperreabsorption of $Ca^{2+}$, and global defects in cellular $Ca^{2+}$-ATPase activities. The latter hypothesis was tested by measuring $Ca^{2+}$-transport activity in erythrocytes and its correlation with serum calcium and serum parathyroid hormone levels in patients with FBH or primary hyperparathyroidism. The results have shown that some other mechanism than a global defect in $Ca^{2+}$-ATPase activity is responsible for the hypercalcemia in patients with FBH (171).

A significantly increased level of kidney calcium in parallel with an elevated level of $Ca^{2+}$-ATPase activity has been observed in kidney basolateral membranes in rats after 2 to 7 days of high-saline ingestion. This activity was not directly affected by NaCl, parathyroid hormone or calcitonin. On the other hand, the expression of mRNA for the $Ca^{2+}$-binding protein regucalcin was markedly suppressed, suggesting that saline ingestion causes the disturbance of the $Ca^{2+}$

transport system in the kidney cortex of rats, and that the renal disorder may induce hypercalcemia (172).

Hypercalciuria, hypomagnesia and bone mineral loss due to altered divalent cation homeostasis have been associated with human diabetes mellitus. As shown in a diabetic model, rats made diabetic by chronic (2 weeks) steptozotocin administration did not present any changes in the levels of plasma membrane $Ca^{2+}$ pump in whole kidney. Thus, the findings support the notion that PMCA pump in kidneys plays only a marginal role in the altered divalent cation homeostasis in diabetes (173).

Proteinuria is a disorder correlated with interstitial inflammation and fibrosis, hypertension and with progression to end-stage renal disease. In response to excessive protein levels, the tubular epithelium releases factor(s) which correlate with renal insufficiency and may contribute to some of these complications. Proteinuric urine contains a small lipidic factor that acts as a powerful specific inhibitor of the $Ca^{2+}$ pump of red blood cells. This inhibitor had little or no effect on the $Na^+$-$K^+$-ATPase (174). The factor is present in the circulation since hemodialysis returns the activity to normal levels. Because the $Ca^{2+}$ pump is ubiquitous, an inhibitor secreted into the urine may affect other cells and contribute to the progress of the renal disease. When reaching the circulation it may exert systemic effects on target cell $Ca^{2+}$ homeostasis like smooth-muscle cells and cause hypertension.

Circulating inhibitors of the plasma membrane $Ca^{2+}$ pump have been identified also in chronic renal failure. Chromatography and mass spectrometry of ultrafiltrate identified three factors all of which were sufficiently lipophilic to penetrate the blood-brain barrier and to accumulate in cerebral tissue (175). All factors were additive in their inhibitory effect on PMCA activity with a non-competitive mechanism of action. These inhibitors may play a role in the pathophysiology of cellular $Ca^{2+}$ metabolism in renal failure. In very recent work Jankowski *et al.* (176) have characterized an inhibitor as p-hydroxy-hippuric acid. Erythrocyte $Ca^{2+}$-ATPase has been shown to be inhibited by this compound at a concentration above 11.7 μmol/L by reducing the $V_{max}$ and increasing the $K_m$ value.

Adenosine, a potent autacoid produced and released in the kidney, affects nearly all aspects of renal function. In addition, an increase in $[Ca^{2+}]_i$ has been implicated in adenosine effects. Adenosine exerts a biphasic influence on the $Ca^{2+}$-ATPase activity of basolateral membranes of kidney proximal tubules. Inhibition occurs at up to 0.1 μM and then gradually disappears. The effect is mediated by both A1 (inhibitory) and A2 (stimulatory) receptors. By using toxins, it was shown that a G-protein mediated pathway is involved that also encompasses a phospholipase C and a protein kinase C (177).

In uremic patients, the $Ca^{2+}$ content in erythrocytes is elevated. Shalev (178) has shown that the $Ca^{2+}$-ATPase activity of the erythrocytes is decreased. It is suggested that the increased $[Ca^{2+}]_i$ found in other tissues in uremia is the result of the systemic malfunction of PMCAs.

### 7.5. Abnormalities of PMCA function in hypertension

The cell types most directly involved in the hypertensive state are vascular smooth-muscle cells and cardiac muscle cells. In addition to altered contractile properties, blood vessels from hypertensive animals present an increased wall thickness. Because $[Ca^{2+}]_i$ regulates both tension and growth, altered $Ca^{2+}$ levels have been a focus of attention in several laboratories. When an increase of $[Ca^{2+}]_i$ has been detected, the question has been addressed whether this is caused by an increased $Ca^{2+}$ influx or a decreased efflux. In the first case, any increase of PMCA expression could be the result of a compensatory mechanism. In the latter case, a decrease of PMCA function could be a causative factor.

The canine vascular wall contains at least two types of smooth-muscle cells: type 1 cells express muscle-specific proteins and do not proliferate in culture, type 2 cells do not express muscle-specific proteins and proliferate in culture. Both cell types express PMCA1 and PMCA4b. PMCA4a is absent from type 2 cells but it is upregulated in these cells upon placement in culture. The upregulation is inhibited by phosphoinositide 3-kinase inhibitors (179).

Both elevated $Ca^{2+}$-pump-mediated $Ca^{2+}$ efflux and elevated PMCA1 and SERCA2 mRNA levels have been observed in cultured aortic smooth-muscle cells from spontaneously hypertensive rats compared to those from the normal Wistar strain (180, 181). Angiotensin II increased the level of PMCA1 and SERCA2 mRNA in both strains. This study provided evidence for altered $Ca^{2+}$ homeostasis in the spontaneously hypertensive rat model affecting $Ca^{2+}$ transport ATPases at the transcriptional and functional level. It is also consistent with the hypothesis on the connected expression of these two $Ca^{2+}$ pumps (182).

In spontaneously hypertensive rats and hereditary cardiomyopathic hamsters, activities of both plasma membrane $Ca^{2+}$-ATPase and $Na^+$-$K^+$-ATPase in crude myocardial homogenates have been shown to be depressed to 50 and 26 percent respectively, compared to age-matched healthy animals (183). The decrease was selective in relation to overall protein content. Reduced $Na^+$-$K^+$-ATPase and $Ca^{2+}$-ATPase activities may thus be important for the development of heart failure and arrhythmia in hypertrophic heart disease. In a recent study with human subjects, however, mRNAs for both the α2 and α3 subunits of $Na^+$ pump as well as for PMCA isoforms were significantly increased in the right atrium of hypertensives compared to control subjects.

This suggests a compensatory mechanism to overcome defective ion metabolism in hypertensives (184).

A dysregulated $Ca^{2+}$ homeostasis in other tissues than smooth or cardiac muscle, could point to a general causative defect contributing to the pathogenesis of hypertension. Such studies have mainly been conducted on red blood cells. Work on human hypertensive subjects yielded controversial results, with either a decreased or unaltered $Ca^{2+}$-ATPase activity and/or $Ca^{2+}$-pump protein density (180, 185 - 188).

The consequences of other pathologies may add up with hypertension in affecting plasmalemmal $Ca^{2+}$-ATPase activity. Diabetes mellitus type 1 in combination with hypertension has been shown to further reduce $Ca^{2+}$-ATPase activity. This effect was more pronounced in women than in men (133). Interestingly, treatment of hypertension by angiotensin converting enzyme inhibitors causes a long-term enhancement of the pump activity of erythrocytes (189).

In children of essential-hypertensive patients, erythrocyte $[Ca^{2+}]_i$ is elevated. These children also showed decreased erythrocyte $Ca^{2+}$-ATPase activities, suggesting that decreased PMCA function may be one of the mechanisms (190). Parental hypertension was also shown to be a predictor of reduced erythrocyte $Ca^{2+}$-ATPase activity (133).

Studies on synaptosomes revealed reduced $Ca^{2+}$-transport activity mediated by PMCA in spontaneously hypertensive rats (191). In addition, requirements for several volatile anesthetics were also markedly reduced in these hypertensive animals.

A positive correlation has been found in hypertensive black South-Africans between hypertension and the intracellular $Ca^{2+}$ in erythrocytes and platelets, and a negative correlation with the $Mg^{2+}$ and $K^+$ concentration in plasma. In line with these findings, it has been demonstrated that platelet and erythrocytic $Na^+$-$K^+$-ATPase and $Ca^{2+}$-ATPase activities were significantly depressed, which probably reflects a membrane abnormality in hypertensives (192).

By studying the expression of SERCA and PMCA isoforms in platelets from hypertensive rats, Martin *et al.* (193) have shown that hypertension modulates the expression of mRNA of PMCA and SERCA in an opposite direction. Hypertension induces a decrease of PMCA1b expression and an increase of PMCA4b mRNA. Concomitantly SERCA3b/3c is downregulated and SERCA3a increases in this pathology. Hence, PMCA and SERCA proteins should be regarded as constituting a new rational basis for the understanding of non-muscle cell $Ca^{2+}$ signaling especially in platelets in hypertension.

Hypertension is a risk factor for thrombotic events. The early reports have shown that human megakaryoblastic cells (platelet precursor cells) and mature platelets express PMCA1, PMCA2 and PMCA4 isoforms (194). However, using more specific antibodies, it has been recognized that platelet precursor cells express also isoform hPMCA1b which is of smaller size than that seen in HeLa and COS-7 cells, indicating the presence of a modified form of hPMCA1b. In mature platelets, no evidence of the expression of hPMCA1b could be found. In general, the amount of all PMCA forms in precursor cells decreases dramatically during maturation and is lower than SERCA2b or the amount of PMCA in human erythrocytes (195). Platelets of hypertensive individuals exhibit both increased $[Ca^{2+}]_i$ and enhanced sensitivity to agonists. It was demonstrated that platelet PMCA $Ca^{2+}$-ATPase activity inversely correlates with diastolic blood pressure and that inhibition of this $Ca^{2+}$ pump could explain the elevation of cytosolic $Ca^{2+}$ in hypertension. Interestingly, it was recently shown that platelet PMCA can be phosphorylated on tyrosine residues thereby inhibiting the activity. Tyrosine phosphorylation was increased in hypertensive humans, suggesting a causal link with increased platelet $[Ca^{2+}]_i$, hyperactive platelets and increased risk of stroke (196).

Pregnancy produces profound modifications of the platelet plasma membrane, one of which is an enhanced PMCA $Ca^{2+}$-ATPase activity. Hypertension induced by pregnancy (PIH) further enhances PMCA $Ca^{2+}$-ATPase activity of maternal platelets. In addition, PIH increases membrane fluidity due to alteration of the ratio between unsaturated and saturated fatty acids (197).

One of the polymorphism's of the PMCA1 gene in humans has been investigated for a possible correlation with essential hypertension. T to G transversion has been identified resulting in the substitution of a methionine by an arginine at amino-acid position 267 in a highly conserved domain of the pump molecule. Neither this mutation nor differences in single-strand conformation polymorphism (SSCP) or in heteroduplex (HTX) analysis could be identified between hypertensives and normotensives groups (198, 199).

This survey has shown that PMCA levels are altered in hypertension. A decrease was consistently observed in erythrocytes, and in most studies also in platelets. However, PMCA levels were increased in cultured smooth-muscle cells of the vascular wall and in cardiac atrium. Because smooth-muscle cells are intimately involved in the regulation of blood pressure, the work on cultured cells needs to be supplemented with experiments on freshly isolated tissue samples.

### 7.6. Disturbances in vitamin D dependent PMCA modulation

In the intestine, transcellular transport of calcium probably involves three steps: i) passive $Ca^{2+}$ entry into the enterocyte ii) translocation of $Ca^{2+}$ through its cytosol, probably bound to the $Ca^{2+}$-binding protein calbindin iii) active extrusion of $Ca^{2+}$ across the basolateral membrane by an active transport process driven by ATP (mediated by PMCA pumps). Human and rat intestinal mucosal cells contain predominantly the PMCA1b isoform. Reflecting the capacity of the different small-intestinal segments to transport $Ca^{2+}$, much higher levels of transcript were detected proximally (in duodenum) than distally (in jejunum and ileum). Levels were also higher in caecum and ascending colon mucosa than in descending colon (200). These data confirm the importance of the transcriptional regulation of PMCA genes (201).

The vitamin D dependency of the overall absorption of $Ca^{2+}$ is well documented. In addition to vitamin D status, the capacity of $Ca^{2+}$ absorption is influenced by dietary intake of $Ca^{2+}$ and phosphorus. Vitamin D replenishment is linked with significant increase of PMCA pumps and calbindin $D_{28K}$, a vitamin D dependent $Ca^{2+}$-binding protein, in mucosal cell by a factor of 2-3. Likewise, it seems that dietary variables that enhance $Ca^{2+}$ absorption also increase the amount of PMCA pump protein (202, 203).

$Ca^{2+}$ absorption declines with age. This is due to decreased expression of the plasma membrane $Ca^{2+}$ pump mRNA and protein, along with calbindin mRNA and protein, and the declining response of $Ca^{2+}$ absorption to vitamin $D_3$ with age (204). In rats, the decline is substantial and amounts to a decrease by 90 percent in the duodenum and 65 percent in the ileum between 2 and 12 month of age. In addition, the capacity of vitamin D to increase PMCA pump protein is decreased by 61 percent in the adult compared to young animals, which correlates with the decreased capacity of vitamin D to stimulate $Ca^{2+}$ transport in adults. The expression level of $Ca^{2+}$ influx channel (epithelial calcium channel ECaC) of the apical membrane was found to be variable but to correlate closely with that of the PMCA pump. This may contribute to the individual variability in calcium absorption in humans (205).

Horst *et al.* (206) compared the relative effect of $1,24(OH)_2D$ vitamin $D_2$, $1,24(OH)_2$vitamin $D_3$ and $1,25(OH)_2$vitamin $D_3$ in 7-week-old rats on duodenal PMCA mRNA. All metabolites elevated the mRNA levels to a similar extent.

The regulation of PMCA expression by vitamin D is generally present in transporting epithelia such as in intestine, kidney and bone mesenchymal cells. mRNA levels of calbindin D, a vitamin D3 dependent $Ca^{2+}$-binding protein 28k ($CaBP_{28K}$), PMCA1 and PMCA4 have been studied in distal convoluted cells in hypertensive and normotensive Wistar-Kyoto rats. PMCA2 and PMCA4 were not different in the two strains. However, at the protein level, calbindin-$D_{28K}$ protein was more abundant, and PMCA protein was decreased in hypertensives, suggesting that the decreased PMCA level in distal cells may be responsible for the hypercalciuria in hypertensive rats (207).

In addition to vitamin D, prolactin has been proposed as calcium regulating substance during pregnancy and lactation. Prolactin directly stimulates $Ca^{2+}$ transport in duodenum of female rats. Because the effect is inhibited by a calmodulin antagonist at the serosal side, PMCA activity could be involved in the effect (208).

### 7.7. PMCA modulation in other calcium transporting tissues

The influx of large quantities of calcium and phosphate in the enamel and dentin matrices of developing teeth suggests that the formative cells may have a regulatory role in the mineralization processes. Ameloblasts which border the enamel-forming surface undergo complex changes during the secretion and enamel maturation stages in which enamel acquires the bulk of its mineral content. While the enamel forming cells supply calcium, they must avoid the cytotoxic effects of excess $Ca^{2+}$. Disrupted $Ca^{2+}$ transport could contribute to a variety of developmental defects in enamel. The molecular machinery is a potential target for drugs to improve enamel quality (209). The presence of PMCA has been cytochemically demonstrated in both secreting and maturating ameloblasts. An immunological study of (210) suggests a close correlation between the progression of mineralization of enamel and dentin and the amount of epitopes of PMCA pump protein. In addition to regulation of intracellular $Ca^{2+}$, PMCA may thus play a role in the regulation of the delivery of $Ca^{2+}$ to the mineralizing enamel and dentin.

Chronic adult periodontitis is known to produce cytokines in response to changing levels of bacterial lipopolysaccharide (LPS). In gingival fibroblasts, changes in PMCA content alter several $Ca^{2+}$-dependent activities, including the production and release of cytokines. Fibroblasts from chronic adult periodontitis patients exhibited significantly lower levels of $Ca^{2+}$-pump protein than fibroblasts from healthy subjects. However, bacterial LPS alone did not affect significantly the amount of PMCA protein. This suggest that molecular factors other than LPS may be involved in the specific down-regulation of the $Ca^{2+}$ pump in periodontitis (211).

Also in bone–forming cells several PMCA isoforms are expressed (212 - 214).

During lactation, the mammary gland transports large amount of $Ca^{2+}$ from the blood to milk via alveolar

cells. The $Ca^{2+}$ content of most extramammary tissue increases more than 12 fold at the time of initiation of lactation. The control of $[Ca^{2+}]_i$ essential to normal cell function requires the concerted effort of several $Ca^{2+}$-ATPases. Three mRNAs for plasma membrane $Ca^{2+}$-ATPases (PMCA1b, PMCA2b and PMCA4b) and two mRNAs for SERCAs (SERCA2 and SERCA3) and the rat homologue to the yeast Golgi $Ca^{2+}$-ATPase have been identified by RT-PCR analysis. Generally, transcripts increased from the 7th day of pregnancy to the 14th day of lactation. The PMCA2b is the most abundant transcript and at the peak of lactation its levels approached that of actin. The presence of PMCA2b in lactating glands is of particular interest, since this isoform was observed in significant amount only in brain and nervous tissue. The very high affinity of PMCA2 for $Ca^{2+}$ and calmodulin indicates that like in brain, PMCA-mediated $Ca^{2+}$ extrusion is extremely effective also in mammary glands (215).

Similarly to kidney and intestine, PMCA immunolabeling is conspicuous in basal (fetal facing) layers of placental syncytiotrophoblasts, a site of translocation of large amounts of $Ca^{2+}$ (216). The ratio of $calbindin_{9K}$ mRNA level to beta actin mRNA increased 135 fold during gestation in rats and was associated with materno-fetal clearance of calcium (217). However, the ratio of PMCA to actin rose only two to three times. Thus, its seems that expression of the $calbindin_{9K}$ but not PMCA is rate-limiting for placental $Ca^{2+}$ transport in the rat. During gestation in humans, however, PMCA levels did not change. There was however a clear activation of $Ca^{2+}$ transport in isolated basal membrane vesicles (218). Placental trophoblasts from preeclamptic women express a 50 percent lower $Ca^{2+}$-ATPase activity compared to normotensive pregnant women (219). The pathophysiological meaning of this observation is, however, not clear.

PMCA protein is also present in mammalian eye tissue. As documented by immunofluorescent study, in the normal corneal epithelium, both plasmalemmal $Ca^{2+}$-pump and caveolin are located along the cell surface. In the regenerating epithelium after injury, plasmalemmal $Ca^{2+}$-pump stains as many dots in the cytoplasm around the plasma membrane. In contrast, caveolin persisted along the cell surface and remained in caveolae. When the wound closes, the $Ca^{2+}$pump regains its normal distribution. This indicates that caveolae undergo compositional modification during the healing process and that the phenomenon may be related to possible fluctuations of $[Ca^{2+}]_i$ in the regenerating epithelium (220).

The clarity of the lens is a prerequisite for normal vision and is dependent on maintenance of intracellular $Ca^{2+}$ homeostasis. $Ca^{2+}$-ATPase present in lens epithelium is essential for the removal of cytosolic $Ca^{2+}$. Human lens membranes prepared from cataractous lenses express only approximately 50% of the $Ca^{2+}$-ATPase activity as measured by [$\gamma$-$^{32}$P]ATP hydrolysis compared to healthy individuals (221). In addition, order in the lipid hydrocarbon chains increased from 55% in clear lens fibers to 84% in cataractous lens fibers, suggesting that membranes are deranged in cataractous tissue probably by oxidation. This should lead to altered levels of $Ca^{2+}$. The hypothesis has been substantiated by *in vitro* oxidation of lens epithelial membranes by the $Fe^{2+}/Fe^{3+}$ and ascorbate system. The level of $Ca^{2+}$-ATPase inhibition and the degree of lipid hydrocarbon chain order correlated well with lipid oxidation and ascorbate concentration. The mechanism of $Ca^{2+}$-ATPase inhibition as probed by SH compounds like glutathione which reverse the inhibition suggests a direct oxidation of protein sulhydryls, not a secondary process involving oxidation-induced lipid structural changes or products of lipid oxidation (222). Binding of lens alpha cristallin *in vitro* to the membrane lipids did not protect the $Ca^{2+}$ ATPase from thermal or oxidative damage (223).

The blood-brain barrier and blood-cerebrospinal fluid (CSF) barrier are involved in the long-term regulation of the concentration of $Ca^{2+}$ and $K^+$ in the whole brain and CSF. The plasma membrane $Ca^{2+}$ pump is located on the apical membrane of the choroid plexus (CSF facing) membrane (224). Only a limited number of papers deal with the role of PMCA in the choroid plexus in pathological states. The importance of PMCA at the blood-CSF and blood-brain barrier has been examined in brain $Ca^{2+}$ homeostasis in dietary acquired hypo- and hypercalcinemia (225). Western blot analysis revealed that the amount of PMCA protein in the choroid plexus did not vary with the plasma $Ca^{2+}$ concentration. In contrast, in cerebral microvessels from hypocalcemic rats a 150 percent increase of PMCA molecules was found. The location of the PMCA at the blood brain barrier has not been firmly established although there is evidence for an abluminal (brain facing) $Ca^{2+}$-ATPase distribution (226), which could suggest that the increase in the amount of PMCA in the microvessels in hypocalcemia would contribute to maintain $Ca^{2+}$ flux from blood to brain.

## 8. ALTERATIONS OF PMCA IN AGING

Biological aging is a fundamental process that results in a progressive decline in cellular function. There is a possible linkage between the energetic costs associated mainly with the maintenance of the intracellular $Ca^{2+}$ gradient necessary for cellular signaling, oxidative damage and the loss of physiological responses associated with aging. Thus, oxidative stress, and the loss of $Ca^{2+}$-regulation are implicated as primary factors associated with degeneration including aging (61). The cellular alterations with progressing age involve mechanisms which are still unresolved.

### 8.1. Effect of aging on PMCA in neuronal cells

In a series of papers Michaelis´s group has described that as a function of aging both the activity

and membrane levels of the brain PMCA $Ca^{2+}$-ATPase are significantly reduced in both Fischer 344 and the longer lived Fischer 344/Brown Norway hybrid rats (227, 228). In addition, PMCA pump activity from 34 month old animals showed a decrease of $V_{max}$ from 60.9 to 32.4 nmol/mg/min with no change in affinity for $Ca^{2+}$ partly due to a decreased level of calmodulin and its ability to stimulate PMCA activity in old animals (229).

The PMCA pump is very sensitive to inhibition by RO(N)S *in vitro* and hence may be a target of oxidative stress in the aging brain. In addition, the loss of PMCA immunoreactivity after exposure to $ONOO^-$ suggests that this radical induces fragmentation of the PMCA protein (47). Since the increase in hydrophobic interactions, carbonyl groups and nitrotyrosine formation have been reported for various proteins in aged tissues, it is possible that increased oxidative stress in the aging brain can also lead to modifications of PMCA proteins. Similarly to the effect of *in vitro* oxidative stress (47), a decrease of the $Ca^{2+}$-ATPase activity has been observed in aged rats (230). Calcium content in brain tissue was also significantly raised in aged rats (50 weeks) as compared to young animals (5 weeks). As shown by using SH-group modifying factors there were no differences in extent of $Ca^{2+}$-ATPase activity inhibition between aged and young groups, suggesting that SH groups in active sites of the enzyme are probably not impaired in aging. Interestingly, also the ability of the $Ca^{2+}$-ATPase to respond to signaling factors such as cAMP and calmodulin, decreased or disappeared. The findings at least partially explain aging-induced mechanisms causing calcium accumulation in brain (230).

As reviewed recently, post-translational structural changes of critical $Ca^{2+}$-regulatory proteins such as calmodulin have been identified which result in the inability to activate a range of target proteins, including the PMCA pump (61). Oxidation of multiple methionines to corresponding methionine sulfoxides within calmodulin during aging leads to a nonproductive and conformationally disordered complex with PMCA protein. In contrast to the results with calmodulin obtained from senescent Fischer 344 rats, calmodulin isolated from the long-lived Fischer 344/BNF1 is not oxidatively modified in animals that are 34 months old. In addition, in Fischer 344 rat brain there is a large decrease in the function of the 20S proteasome, which *in vitro* selectively degrades oxidized calmodulin. Thus, these findings indicate that structural and functional changes of calmodulin and PMCA protein are consistent with the observed decreases in $Ca^{2+}$ regulation in senescent animals (61, 231).

In the hippocampus of senescence-accelerated P8 mice, the levels of protein kinase C gamma and of calbindin decline markedly with age. However, the level of total PMCA molecules did not change significantly with age (232). Because in this study antibodies were used which recognized all PMCA isoforms, the possibility that individual isoforms do change with age is not excluded.

Our unpublished data also indicate possible conformational alterations of the protein molecules or age-related protein modifications of the ion-transport ATPases which may contribute to age-induced changes in hippocampal function.

### 8.2. Effect of aging on PMCA in other cell types

A general response to aging in myocardial cells is a prolonged excitation-contraction coupling cycle. At the transcriptional level, the density of the SERCA $Ca^{2+}$ pump was reduced as a consequence of the down-regulation of the gene (233). Only one paper deals with the effect of aging on PMCA in myocardial cells. Frolkis *et al.* (234) in a study of myocardial hypertrophy induced by aorta coarctation proposed a role of molecular intracellular regulators which increase the activities of the $Na^+$-$K^+$-pump or the plasmalemmal $Ca^{2+}$ pump. They showed in adult rats (6 - 8 months) that coarctation induces an increase of $Na^+$-$K^+$-ATPase and $Ca^{2+}$-ATPase activities, whereas in old rats only the $Ca^{2+}$-ATPase is affected. Because the effects can be mimicked by addition of isolated cytosol to control membranes, the authors propose that aorta coarctation induces the synthesis of activators in adult rats. Because cytosol of old animals after coarctation did not activate the $Na^+$-$K^+$-ATPase, but was shown to activate the $Ca^{2+}$-ATPase, the authors suggested the occurrence of two different regulators.

## 9. CONCLUSIONS AND PERSPECTIVES

$Ca^{2+}$ homeostasis is crucial for cell function and survival. Local $Ca^{2+}$ signalling requires systems for both the entry and the removal of $Ca^{2+}$ maintained under precise spatial and temporal control. Mechanisms that regulate cellular $Ca^{2+}$ have been studied extensively, and remarkable progress has been made in understanding their roles also in pathological conditions. Although the general function of PMCA is well documented, much less is known about the role of specific PMCA isoforms, their spatial organization and especially their contribution to the onset and progress of pathological conditions.

Up till now no human disease has been linked to a defect in any of the PMCA genes, which could be indicative of a high degree of redundancy of the functions of each isoform. However, molecular biology techniques have given striking examples of the indispensability of specific gene products for several physiological functions. The PMCA2 isoform in the ear is a striking example.

Alterations of PMCA isoform expression at the transcriptional level have been described in response to

a variety of factors, among them $Ca^{2+}$ itself, vitamin D and steroids. In $Ca^{2+}$-transporting epithelia, PMCA expression is increased by vitamin D. PMCA expression in these cells decreases with age, as does the response to vitamin D. Effects of steroids have been observed in the brain and neurons in culture. $Ca^{2+}$ itself affects the expression of PMCA2 and PMCA3 in neurons and this effect appears to be an essential component in differentiation and survival following $Ca^{2+}$ overload. Alterations of PMCA transcription may also occur in hypertension, atherosclerosis and aging.

Non-genetic alterations of PMCA activity may be an important factor contributing to pathological conditions, especially in acute dysfunctions like those initiated by $Ca^{2+}$ overload and/or oxidative stress. In addition, structural and functional modifications of PMCA molecules in cells from different tissues by external or internal oxidative agents may contribute to slowly progressing pathologies such as atherosclerosis, hypertension, degeneration and aging. $Ca^{2+}$ is also an essential component of the pathway leading to cellular death. Proteolysis of PMCAs has been observed in apoptotic conditions. Diabetes mellitus is another condition with impaired $Ca^{2+}$ homeostasis and in which PMCA activity is decreased, not only in pancreatic beta cells but in erythrocytes as well. Oxidative damage to PMCA has been considered as a causative factor for the abnormally high $Ca^{2+}$ content in sickle cell anemia. Abnormalities of the $Ca^{2+}$ pump of erythrocytes often seem to parallel alterations of the pump in other tissues, such that increased erythrocyte $Ca^{2+}$ content and depressed $Ca^{2+}$-ATPase activity is a common response to diverse diseases.

The cell types most directly involved in the hypertensive state are vascular smooth muscle cells and myocardial cells. As there appears to be an increase of $[Ca^{2+}]_i$ in hypertension, an elevation of mRNA and protein for PMCA1 and PMCA4b could be a general response probably as the result of compensatory mechanisms. In cells of other tissues, such as red blood cells and platelets, a depression of $Ca^{2+}$-ATPase activity is observed. This raises the question whether these changes could play any role in hypertension-related risk factors like thrombosis and whether PMCA and SERCA $Ca^{2+}$ pumps can be considered as prospective therapeutic targets.

The regulation of PMCA expression by vitamin D in transporting epithelia is generally observed. PMCA expression and the response to vitamin D decline with age. In addition, the expression and functional state of PMCA protein is influenced by other pathogenic factors such as mineral dysregulation.

Depressed $Ca^{2+}$-ATPase activity in aging may not only occur at the level of the PMCA protein itself, but may also be caused by structural oxidative changes of critical regulatory proteins such as calmodulin.

Investigation of pathology-induced changes in the level and activity of PMCA $Ca^{2+}$ pumps may help to identify patterns that are specific for vulnerable and resistant cells. Knowledge of such a pattern would greatly assist the development of specific therapeutic strategies to activate recovery of $Ca^{2+}$ extrusion and help to establish whether these changes are of relevance to the pathological process leading to cell injury.

## 10. ACKNOWLEDGMENTS

This work was supported in part by Slovak VEGA Grant (7417/20) to J.L.

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**Abbreviations:** PMCA: plasma membrane $Ca^{2+}$ pump, SERCA: sarco(endo)plasmic $Ca^{2+}$ pump, RO(N)S: reactive oxygen(nitrogen) species, IRI: ischemia/reperfusion injury, NMDA: N-methyl D-aspartate, TBARS: Thiobarbituric acid reactive substances

**Key Words:** Neurons, Muscle Cells, $Ca^{2+}$ Transporting Epithelial Cells, Red Blood Cells, Liver, Kidney, Disorders, Ischemia, Oxidative Stress, Diabetes, Hypertension, Aging, Plasma Membrane $Ca^{2+}$ Pump, Modification, Review

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