Intracellular Ca^{2+}- and Mn^{2+}-Transport ATPases

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1. Introduction

The endoplasmic reticulum (ER) and the Golgi apparatus are Ca^{2+}-containing intracellular organelles with a major role in intracellular Ca^{2+} homeostasis. Their Ca^{2+} can be released into the cytosol, from where it controls a range of physiological processes depending on the subcellular localization, the amplitude, and the frequency of the cytosolic $[Ca^{2+}]$ rise.[1] The ER and the Golgi apparatus must at the same time keep their luminal $[Ca^{2+}]$ sufficiently high, since this ion is indispensable for the normal function of these compartments. Lipid membranes in cells form low-permeable barriers that limit the free exchange of ions and polar solutes. They are therefore embedded with a wide variety of proteins mediating the transport of ions and nutrients needed to sustain life. Ca^{2+} is taken up in the lumen via sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCA) and secretory-pathway Ca^{2+}-ATPases (SPCA). The latter pumps also provide the Golgi lumen with Mn^{2+} needed for some biochemical reactions.

This review will first focus on the eukaryotic Ca^{2+}-ATPases of the ER and on the Ca^{2+},Mn^{2+}-ATPases of the Golgi apparatus. They both belong to the P_{2A} branch of the P-type-ATPase superfamily, which separated already in prokaryotes from the P_{2B} class that includes the plasma membrane Ca^{2+}-ATPases (PMCA). We will then review the role of Ca^{2+} and Mn^{2+} in the lumen of the intracellular stores and finally focus on Mn^{2+} toxicity.

2. Phylogenetic Relationship between SERCAs and SPCAs

The P-type ATPases transport ions or molecules across biological membranes using the energy of ATP hydrolysis.[2] The designation P-type comes from the mechanism by which

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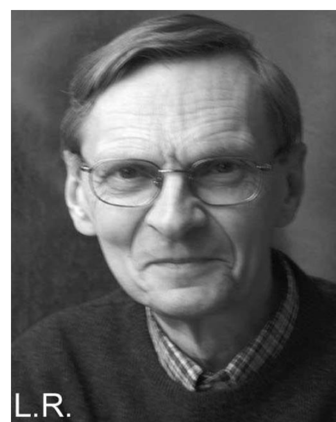
the terminal phosphate of ATP is transiently transferred to a highly conserved aspartate residue in the active site, resulting in reversible conformational changes. Classifications of the P-type ATPases based either on sequence similarity or on substrate specificity largely overlap and fall into five classes and subclasses. The class formed by the Ca^{2+} pumps is designated P_2 and is represented in vertebrate animals by the SERCA, SPCA, and PMCA families. In higher vertebrates, the SERCAs are encoded by three genes (*ATP2A1−3*), the SPCAs are encoded by two genes (*ATP2C1−2*), and the PMCAs are encoded by four genes (*ATP2B1−4*).[3] Ca^{2+} pumps related to each of these gene families are found in most eukaryote species.

Among the Ca^{2+}-transport ATPases, only the SERCA-type pumps are characterized by two high-affinity Ca^{2+}-transport sites, designated site I and site II. Consequently, they can transport two Ca^{2+} ions per ATP hydrolyzed, whereas the PMCA-type pumps and the SPCAs bind only one Ca^{2+} ion (binding to a site corresponding to site II of SERCA). Despite this fundamental difference between SERCAs and SPCAs, their overall sequence similarity clearly demonstrates a *closer* relation than between either of them and the PMCAs.

Ludwig Missiaen (L.M.) became medical doctor in 1984 at the Katholieke Universiteit Leuven. In 1988 he obtained his Ph.D. in Medical Sciences at the Laboratory of Physiology of the Katholieke Universiteit Leuven. From 1989 to 1991, he worked as a postdoctoral researcher at the A.FRC Laboratory of Molecular Signalling (c.o. Dr. M. J. Berridge) in Cambridge (U.K.). He is now full professor in physiology at the Katholieke Universiteit Leuven. Here he is involved in the study of SPCAs.



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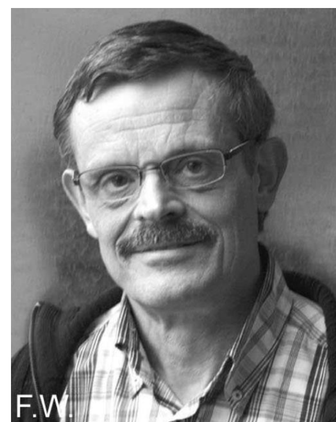

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Therefore, SERCAs and SPCAs are clustered together in a separate subfamily of the P-type ATPases, the P_{2A} branch, whereas the PMCAs fall into the P_{2B} subfamily.[4] The two

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Ca^{2+}-binding sites of SERCAs are formed by 10 amino-acid residues, situated in four of the total 10 membrane-spanning helices: M4, M5, M6, and M8. The Ca^{2+} ions are coordinated either to the side chains or to backbone oxygen atoms (see below). Because of the greater complexity of a two-Ca^{2+} transporter, it appears reasonable that SERCA-type pumps evolved from a simpler single-site ancestor. The most likely picture that then emerges is one where SERCA-type pumps evolved from SPCA-type pumps, after an earlier branching off from a line that gave rise to the extant P_{2B} (PMCA) ATPases. These evolutionary steps must have pre-dated the emergence of the eukaryotes since all amino acids involved in Ca^{2+} coordination in SERCAs are conserved in some bacterial species, as can be seen from the amino-acid alignment of parts of the Ca^{2+}-binding transmembrane segments (Figure 1 top). All 10 residues are conserved, e.g., in *Legionella pneumophila* and *Haloarcula marismortui*, while in other species, the Glu in M8 that forms part of site I is replaced by a Gln, e.g., in *Bacillus subtilis*. Since this SERCA-type Ca^{2+}-coordinating configuration is found in both the oldest branches of life, Eubacteria and Archaea, the branching of SERCAs from SPCAs must have occurred already in very early stages of evolution. Indeed, SPCA-like as well as PMCA-like sequences can be found in both Eubacteria and Archaea, as shown by the family tree in Figure 2 and more convincingly by the alignment of core Ca^{2+}-binding regions that allow the identification of short signature sites (Figure 1). Moreover, within the Eubacteria, sequences belonging to each of the Ca^{2+}-pump branches are found in diverse classes of organisms. The occurrence of both SERCA-type and SPCA-type sequences in the Archaea has been mentioned by De Hertogh et al.[5]

The sequences aligned in Figure 1 fall into three groups, from top to bottom, SERCA-like, SPCA-like, and PMCA-like. On top of each group, the eukaryote consensus sequences are shown. The assignment of the bacterial sequences to one of the three groups is mainly based on the highlighted residues, which are either specific for one group or conserved between SERCAs and SPCAs or between SPCAs and PMCAs. Some of the amino acids highlighted in Figure 1 are not directly involved in Ca^{2+} coordination. However, the significance of these residues is underscored by their conservation not only between paralogous and homologous genes in higher eukaryotes but also in many prokaryote sequences. These residues may therefore contribute in an essential way to the structure of the Ca^{2+}-binding pocket. Indeed, the affinity and the ion specificity of the binding sites is not only determined by the nature of the ion-coordinating residues but also by their spatial constellation, and thus by the global structure of the binding region. This explains why, in the Na^+,K^+-ATPase, a P-type ATPase of the P_{2C} class, with its very different ion specificity and stoichiometry, there is little sequence conservation in the region surrounding the ion-binding sites, while 7 out of the 10 ion-coordinating residues of SERCA are identical. The importance of noncoordinating amino acids is further underscored by mutations and structural analysis.[6,7] Together with the site II Ca^{2+}-coordinating amino acids, the residues highlighted in M5−M6 of SPCA and PMCA could have been conserved from the structure of the putative ancestral ion-binding region.

The family relationships derived from the comparison of the Ca^{2+}-binding sites as depicted in Figure 1 are generally confirmed by the alignment of the entire ATPase chain, as shown in Figure 2. Some bacterial sequences occupy an intermediate position between PMCAs and SPCAs, e.g., the Ca^{2+} pump of *Clostridium*, or between SPCAs and SERCAs, e.g., that of the *Haloarcula* pump. The inclusion of *Clostridium* in the SPCA cluster and of *Haloarcula* in the SERCA group is therefore based on the signature sequences indicated in Figure 1.

It should be kept in mind, however, that the validity of this picture of very early diversification depends on the assumption that prokaryote species did not at later stages acquire Ca^{2+}-pump genes from distant taxa by horizontal transfer.

At present little is known on the role of Ca^{2+} in prokaryotes. Like the eukaryotes, bacteria also maintain a cytosolic [Ca^{2+}] in the submicromolar range, probably because higher concentrations are not compatible with essential life processes. The steep inwardly directed transmembrane Ca^{2+} gradient is maintained by Ca^{2+}/H^+-exchangers, Na^+/Ca^{2+}-exchangers, and possibly by the P-type Ca^{2+} pumps described above. Whether the cytosolic [Ca^{2+}] is dynamically regulated to serve signaling functions as in eukaryotes is less clear.[8,9] Ca^{2+} ions may be involved in the maintenance of cell structure and motility and in differentiation processes. A clear example of the latter is sporulation, which occurs in specific classes of bacteria. Spores accumulate large amounts of Ca^{2+}. In *B. subtilis*, a SERCA-type Ca^{2+} pump is expressed during sporulation. Its knockdown affects the quality of the spores, but apparently it is not required for spore formation.[10] It is, thus, not obvious why P-type Ca^{2+} pumps are present in some bacterial species and not in others, assuming in the first place that the proteins encoded by all these genomic sequences are actually expressed in a functional form. It is, therefore, even more difficult to speculate on possible functional reasons for the evolution and the specific expression of one or more of the three different types of Ca^{2+} pumps in prokaryotes.

The protist phylum Apicomplexa comprises a large group of eukaryotes, which branched off from other eukaryotes at an early point and which is related to ciliates and dinoflagellates. This phylum includes a number of human and animal parasites. Recent whole-genome analyses of representatives of the phylum Apicomplexa including *Plasmodium*, *Cryptosporidium*, and *Toxoplasma*, all of which are human parasites,

		M4	M5	M6	M8
		II II II II	I I	II I II	I
TYPE P$_{2A}$ (SERCA-like)					
SERCA consensus	animals	302 LAVAAIPEGLP/	759 QFIRYLISSNVGEVVSI/	790 VQLLWVNLVTDGLPATALGF/	903 VLVTIEMLNAIN
ECA consensus	plants	LAVAAIPEGLP/	/AFIRYMISSNIGEVASI/	/VQLLWVNLVTDGPPATALGF/	/VLVAIEMFNSLN
Neurospora crassa (1)	fungi	LGVAAIPEGLA/	/QFIRYLISSNIGEVVSI/	/VQLLWVNLVTDGLPATALSF/	/ILVVIEMFNAMN
Bacillus subtilis (2)	Gram pos.	LAVAAIPEGLP/	/KFIRYLLASNVGEILVM/	/IQILWVNLVTDGLPAMALGM/	/TLVLAQLIHVFD
Synechococcus elongatus (3)	Cyanobact.	LAVAAIPEGLP/	/KFIKYILGSNIGELLTI/	/LQILWMNLVTDGIPALALAV/	/TLCLAQMGHAIA
Legionella pneumophila (4)	Proteobact.	LAVAAIPEGLP/	/KTLAYLLAGNSGELLVV/	/IQLLWINLVTDGLPAIGLAT/	/VLVTAELLWAFG
Haloarcula marismortui (5)	Archaea	LAVAAVPEGLP/	/KFVGYLLSANVAEVAIV/	/VQLLWINLLTDGLPALALGA/	/GFVFLEFEKLYV
TYPE P$_{2A}$ (SPCA-like)					
SPCA consensus	animals	LAVAAIPEGLP/	/NFVRFQLSTSVAALSLI/	/MQILWINIIMDGPPAQSLGV/	/CFVLFDMWNALS
Pmr1p *S. cerevisiae* (6)	fungi	LAVAAIPEGLP/	/NFLTFQLSTSVAALSLV/	/MQILWINILMDGPPAQSLGV/	/CFVFFDMFNALA
Azoarcus sp. (7)	Proteobact.	LAVAAIPEGLP/	/KFVRFQLSTNIGAILTV/	/IQLLWINIIMDGPPAMTLGI/	/TFVLFQFFNVFN
Clostridium novyi (8)	Gram pos.	LIVAAVPEGLP/	/RFIQFQITVNIVAFLTA/	/IQLLWVNIIMDGPPALSLGL/	/LFAFNALFNAFN
Bifidobacterium longum (9)	Actinobact.	LIVAAVPEGLP/	/RFIQFQLTVNLSSVVVV/	/LQLLWVNIIMDGPPALTLGM/	/LFVVFQLFNAFN
Methanobrevibacter smithii (10)	Archaea	LAVAAIPEGLP/	/RFVKFQVSTNVGAILTI/	/VQLLWINIVMDGPPAQTLGM/	/LFVMYQLFNAYN
TYPE P$_{2B}$					
PMCA consensus	animals	VLVVAVPEGLP/	/KFLQFQLTVNVVAVIVA/	/VQMLWVNLIMDTFASLALAT/	/TFVMMQLFNEIN
ACA consensus	plants	IVVVAVPEGLP/	/KFIQFQLTVNVAALIIN/	/VQLLWVNLIMDTLGALALAT/	/IFVFCQVFNEFN
Pmc1p *S. cerevisiae* (11)	fungi	VIVVAVPEGLP/	/KFIQFQLIVNITAVILT/	/VQLLWINLIMDTLAALALAT/	/TFVWLQFFTMLV
Synechococcus sp. (12)	Cyanobact.	IIVVAVPEGLA/	/KFLLFQLTINVVALGTA/	/TQMLWVNLIMDTFAALALAT/	/IFVFLQLWNLFN
Akkermansia muciniphila (13)	Chlamydiae	VIVVTVPEGLP/	/RFIVFQMTINVAACLIV/	/TQMLWVNLIMDTFAALALAS/	/IFVLLQFWNMFN
Porphyromonas gingivalis (14)	Bacteroidetes	VIVVAVPEGLP/	/RFILFQMTINVVACIIV/	/TQMLWVNLIMDTFAALSLAS/	/IFVFLQFWNMFN
Methanospirillum hungatei (15)	Archaea	IIVVSVPEGLP/	/RFLIFQLTINISAAILT/	/IQLLWINIIMDSLAALALCS/	/GFVIAQVWNGFN

Figure 1. Sequence alignment of part of the transmembrane regions of Ca^{2+} pumps from higher eukaryotes and bacterial species. Part of transmembrane regions M4, M5, M6, and M8 of Ca^{2+} pumps from higher eukaryotes are aligned with bacterial sequences selected from those that are most similar to the corresponding eukaryote domains. Species were further selected to represent as many as possible of the major taxa. Note that SPCAs are absent in plants. Residues contributing to the formation of Ca^{2+}-binding sites I and II are indicated on top. The Asp in M6 contributes to both sites. The numbers in superscript in the SERCA sequence correspond to the amino-acid numbering in rabbit SERCA1. The type-2A group is split in two parts: SERCA-like and SPCA-like sequences. Residues that are fully or very highly conserved within the eukaryote families, both among paralogous and orthologous genes, are indicated in bold. Bacterial sequences are assigned to one of the three eukaryote classes based mainly on the yellow-highlighted residues. The corresponding residues in SERCA may or may not directly contribute to Ca^{2+} coordination. Amino acids unique to one group are indicated in red. The highlighted serine in M5 of the SPCA-like sequences is unique to eukaryotes. Accession numbers of the species-specific sequences: (1) CAB65295; (2) CAB13439; (3) YP_400099; (4) AAU27103; (5) AAV44847; (6) NP_011348; (7) CAI08472; (8) ABK62185; (9) AAN24843; (10) ABQ87100; (11) P38929; (12) ACB00092; (13) ACD04061; (14) AAQ66072; (15) ABD40112.

indicate the presence in their genomes of two P-type ATPases (PfATPase6 and PfATPase4, both from *Plasmodium falciparum*).[11] Apicomplexans show Ca^{2+}-signaling pathways often with plant-like characteristics.[11]

Plants like *Arabidopsis thaliana* or rice (*Oryza sativa*) express 4 SERCA-type proteins known as ECA ATPases (ER-type Ca^{2+}-ATPase) and 10 ATPases indicated as ACAs (autoinhibited Ca^{2+}-ATPase) that are closely related to the mammalian PMCAs.[12] The latter ATPases contain an autoinhibitory domain, whose inhibitory action on the ATPase is relieved upon binding of calmodulin. The plant ACAs differ from the metazoan PMCAs because their calmodulin-binding domain is positioned at the N-terminus and because they are not always found in the plasma membrane.[13] The plant ECAs[12] can transport both Ca^{2+} or Mn^{2+}. Comparison of the protein sequences and the gene's exon/intron layouts shows that, of all four members of the ECA family in plants, ECA3 appears to be the most closely related to the metazoan SERCA ATPases. ECA1, -2, and -4 together form a separate cluster that shows a much less elaborate exon/intron structure with only 7 introns instead of the 33 introns in the ECA3 gene of *A. thaliana* and *O. sativa*. The close relationship between ECA3 and the SERCAs is further underscored by the observation that, of the 33 introns in ECA3 and the 20 introns in SERCA (introns in the genes encoding SERCA1−3 take similar positions, except for the alternatively spliced ones), 8 are found in corresponding positions. Of note, none of the 7 intron positions in ECA1, -2, and -4 coincide with a corresponding position of the 33 ECA3 introns. Although ECA3 is phylogenetically most closely related to the mammalian SERCAs, it is targeted to the Golgi apparatus and not to the ER as the other three ECAs are and it plays a crucial role in Mn^{2+} homeostasis.[14] ECA3 thus appears to be the functional correlate of the mammalian SPCA in plants, which are otherwise lacking direct orthologues to the SPCA subfamily. Interestingly, in maize (Zea mays), a SERCA-type Ca^{2+}-ATPase has been found that, like PMCAs, is regulated by calmodulin, with its calmodulin-binding domain located at the C-terminus. This Ca^{2+} pump, named CAP1, is apparently unable to transport Mn^{2+}.[15] In fungi and invertebrates, in most lower eukaryotes, and in prokaryotes, the three Ca^{2+}-pump families (SERCA, PMCA, SPCA) are usually represented by at most a single gene per family. Some fungi, e.g., the extensively studied yeast *Saccharomyces cerevisiae*, lack a SERCA orthologue, but most other representatives of the fungi have one.

3. SERCAs

The SERCAs are, together with the SPCAs, responsible for loading the nonmitochondrial intracellular stores with Ca^{2+}. Both pumps belong to the P_{2A}-ATPase phylogenetic subgroup. In humans, 3 genes (*ATP2A1−3*) generate multiple SERCA isoforms (SERCAla,b, SERCA2a−c, and SERCA3a−f) by developmental or tissue-specific alternative splicing.[16] We will first review the structure of the SERCA genes and then discuss the structure of the SERCA protein by focusing on SERCA1a, for which the most information is available. Thereupon we will review the physiological role of the SERCA1, -2, and -3 proteins and end with a discussion of SERCA-specific inhibitors.

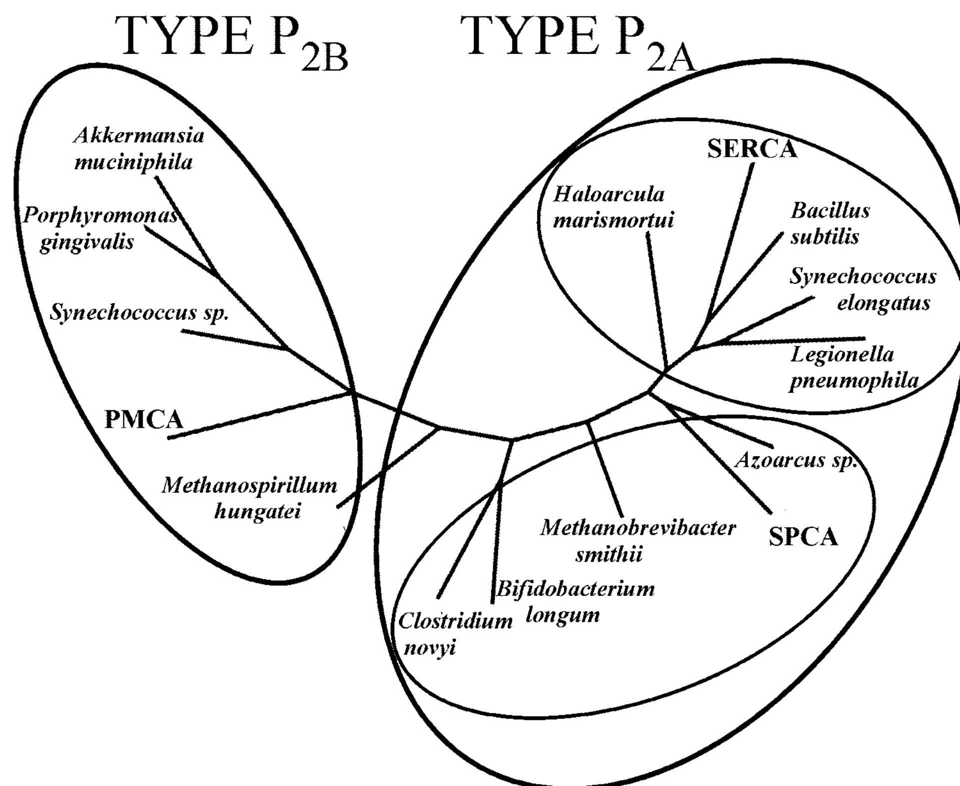

Figure 2. Distance tree of the Ca^{2+} pumps represented in Figure 1. The figure was generated by the Phylogeny program (http://www.phylogeny.fr)[435] using the "One Click" mode. The alignment was done on the full-length amino-acid sequences. Columns in the alignment containing gaps were skipped from further analysis. Eukaryote sequences are represented by PMCA2 of zebrafish (accession no. EU559282), SPCA of *C. elegans* (CAC19895), and SERCA of *C. elegans* (NP_499385). The indicated grouping of the bacterial sequences corresponds to the classification based on the short signature sequences shown in Figure 1.

3.1. Genes encoding SERCAs

Since the SERCA-type of ATPases found in plants were already addressed above, we will here focus on the metazoan SERCAs. All invertebrates, including *Nematostella vectensis*, representing the oldest eumetazoan phylum of Cnidaria,[17] and even *Trichoplax adhaerens*, a very primitive animal belonging to the Placozoa, contain a single SERCA gene in their genome.[18] A notable exception is the parasitic flatworm *Schistosoma mansoni* that has at least two SERCA genes *SMA1* and *SMA2*.[19] As a result of two successive genome duplications, vertebrates typically show 3 SERCA genes. Fish, which witnessed even further genome duplications, have an even larger number of SERCA genes. It is of interest to note that, as discussed earlier,[20] the single SERCA gene found in the invertebrate genome is orthologous to the vertebrate SERCA2 gene (*ATP2A2* in human) representing the housekeeping gene in the vertebrates. With the exception of the introns in the alternatively spliced part of the gene, the positions of the introns in the mammalian genes encoding the three SERCAs (*ATP2A1−3*) are conserved, but they differ from the positions in the SPCA1 and PMCA1 genes. The vertebrates seem to have the most elaborate and primitive intron layout. Invertebrates, including the cnidarian *N. vectensis*, have fewer introns than mammals.[17] The reader is referred to an earlier review for further details on this issue.[20]

3.2. Structure of SERCAs

The first SERCA1a crystal reported in 2000 by Toyoshima et al.[7] was a milestone in our current understanding of the working mechanism of P-type ATPases. Today more than 20 crystal structures in nine different states have been reported, approximately covering the entire reaction cycle.[7,21−30] They serve as a representative model for the ion-transport mechanisms in all P-type ATPases. In fact, more structures of functionally defined conformational states exist for SERCA than for any other membrane protein. The abundant expression of SERCA1a in skeletal muscle facilitated the isolation and purification of the pump. The success of obtaining so many crystals locked in different conformational states should at least partially be attributed to biophysical and biochemical insights in the structure−function relationships of SERCAs gathered over the years.[31]

3.2.1. Architecture of the Pump

Active transport by Ca^{2+}-ATPases is achieved by alternating the affinity and accessibility of the transmembrane Ca^{2+}-binding sites using energy from ATP hydrolysis. These Ca^{2+} pumps consist of two major conformations: E1 and E2. During the reaction cycle, considerable conformational changes take place from the Ca^{2+}-bound E1 form to the Ca^{2+}-free E2 form. In the E1 form, the ion-binding sites display a high affinity for cytosolic Ca^{2+}, whereas in the E2 form, they show very weak affinity for Ca^{2+} and face the lumen.[2,32,33] For each two Ca^{2+} ions transported from the cytosol to the lumen of the ER, 2−3 protons are counter transported, making SERCA an electrogenic Ca^{2+}/H^+-countertransporter.[34,35]

The membrane (M) domain consists of 10 transmembrane helices (M1−10) that contain the ion-binding sites (Figure 3). The large cytoplasmic region is composed of an actuator (A), phosphorylation (P), and nucleotide-binding (N) domain,

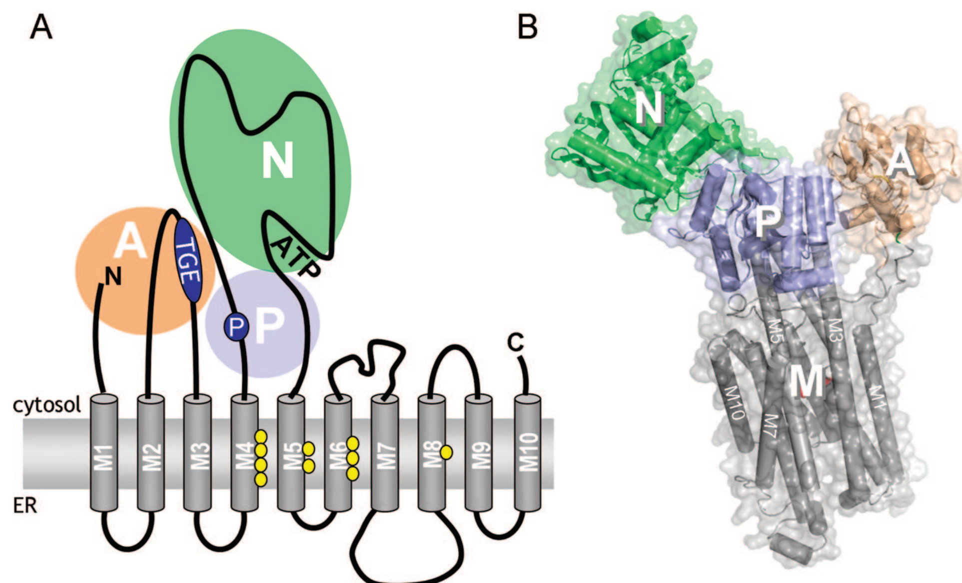

Figure 3. Architecture of the SERCA Ca^{2+} transporter. (A) Planar model of SERCA1a showing the 10 transmembrane helices (M1−M10) and the principal cytosolic domains. Dark blue: the phosphorylation (P) site (Asp351 in SERCA1a) and the dephosphorylation motif ([182]TGE in SERCA1a). Yellow circles indicate the presence of a Ca^{2+}-binding residue. (B) 3D structure of SERCA1a in the E1·2Ca^{2+} conformation (PDB entry 1SU4[7]). Indicated are the actuator domain (A, light brown), the phosphorylation domain (P, light blue), the nucleotide-binding domain (N, green), and the transmembrane region (M, gray).

whereas only small loops appear at the luminal side.[7] The largest cytosolic domain, the N-domain, recruits ATP in its nucleotide-binding pocket.[7] The N-domain is connected to the P-domain via a very conserved, flexible hinge region made up by two antiparallel peptide strands.[7,36] The P-domain contains the highly conserved signature sequence DKTGTLT (with D being the phosphorylation site, Asp351 in SERCA1a), which is common for all members of the P-type ATPase family.[36] This motif catalyzes the autophosphorylation of the P-domain.[37] The A-domain or actuator of the pump is the smallest cytosolic domain.[7] An actuator is by definition a device for moving or controlling a mechanism or system. As will be discussed in more detail, the control function of the A-domain in the pump is complex and differs, depending on its relative position and rotation, throughout the catalytic cycle. The A-domain contains the conserved signature motif TGE, which controls the dephosphorylation reaction.[25]

The distance (∼50 Å) between the catalytic site (phosphorylation site in the cytosol) and the ion-binding sites in the membrane is long,[7] yet communication between the cytosolic domains and the transmembrane region is essential for ion transport. Conformational changes in the P-domain transmit to M3−M6, which are associated with the P-domain.[21] Notably, M5 penetrates deeply into the P-domain and forms the center mast of the enzyme.[7,21] The cytosolic A-domain is connected to the transmembrane region M1−M3 by flexible linkers. Therefore, rotations of the A-domain will exert strain to the connected helices, affecting their relative position.[38] The size of the flexible linkers is critical to ensure proper pump function.[39,40]

3.2.2. Structural Comparison with Other P-type ATPases

Sequence comparison already indicated that the various members of the P-type-ATPase superfamily are structurally related.[36] This assumption is now supported by the comparison of the crystal structures of the plant H^+-ATPase AHA2 (P_3-ATPase branch[41]), the pig Na^+,K^+-ATPase (P_{2C}-ATPase branch[42]), and the rabbit SERCA1a Ca^{2+}-ATPase (P_{2B}-ATPase branch[7]). Although these ion pumps share limited sequence homology (e.g., only 20% between AHA2 and SERCA1a), the overall domain architecture is remarkably similar (Figure 3).[43] As for other protein families with low sequence homology, the structures within the P-type-ATPase superfamily are better conserved than the sequences.[36] This likely reflects conserved mechanisms of transport in all P-type ATPases. Unexpectedly, most residues at corresponding positions in the ion-binding pockets of the Na^+,K^+-ATPase and the ER Ca^{2+}-ATPase are identical, regardless of the size and charge of the ions that they transport.[42] This raises fundamental questions of how the type of ions for transport is selected.[43] Only high-resolution structures at <2 Å would provide sufficient detail to visualize subtle differences in the ion-binding pocket accounting for ion selectivity.

3.2.3. Ion-Transport Mechanism

Studying the structures in different conformations led to a fairly detailed picture of the Ca^{2+}-pumping process. Four principal structures capture the main events of the reaction cycle and are discussed below and depicted in Figure 4 (based on Toyoshima[38]). For a more elaborate description, the reader is referred to recent reviews on the structural aspects of ion pumping.[38,44−46]

Ca^{2+} Entry and Binding: E2 → E1·2Ca^{2+} (Figure 4 D→A). A putative entry pathway for Ca^{2+} is found close to the cytosolic side of M1 and M2 and is flanked by acidic and hydrophilic residues.[44,45,47] From there it leads directly to the Ca^{2+}-binding sites. The two high-affinity Ca^{2+}-binding sites in the transmembrane region of SERCA, sites I and II, are located side by side.[7] These sites are deeply buried in the interior of the pump's transmembrane region, isolated from the lipid environment. Both sites can form seven oxygen coordinations with the Ca^{2+} ion,[7] which is characteristic for a high-affinity Ca^{2+}-binding site.[48] Site I is formed by side-chain oxygen atoms of residues on M5, M6, and M8 and two water molecules (Figures 1 and 5C). The partially unwound M4 and M6 provide all residues for Ca^{2+} binding in site II[7] (reviewed in ref 49). Only residue Asp800 in M6

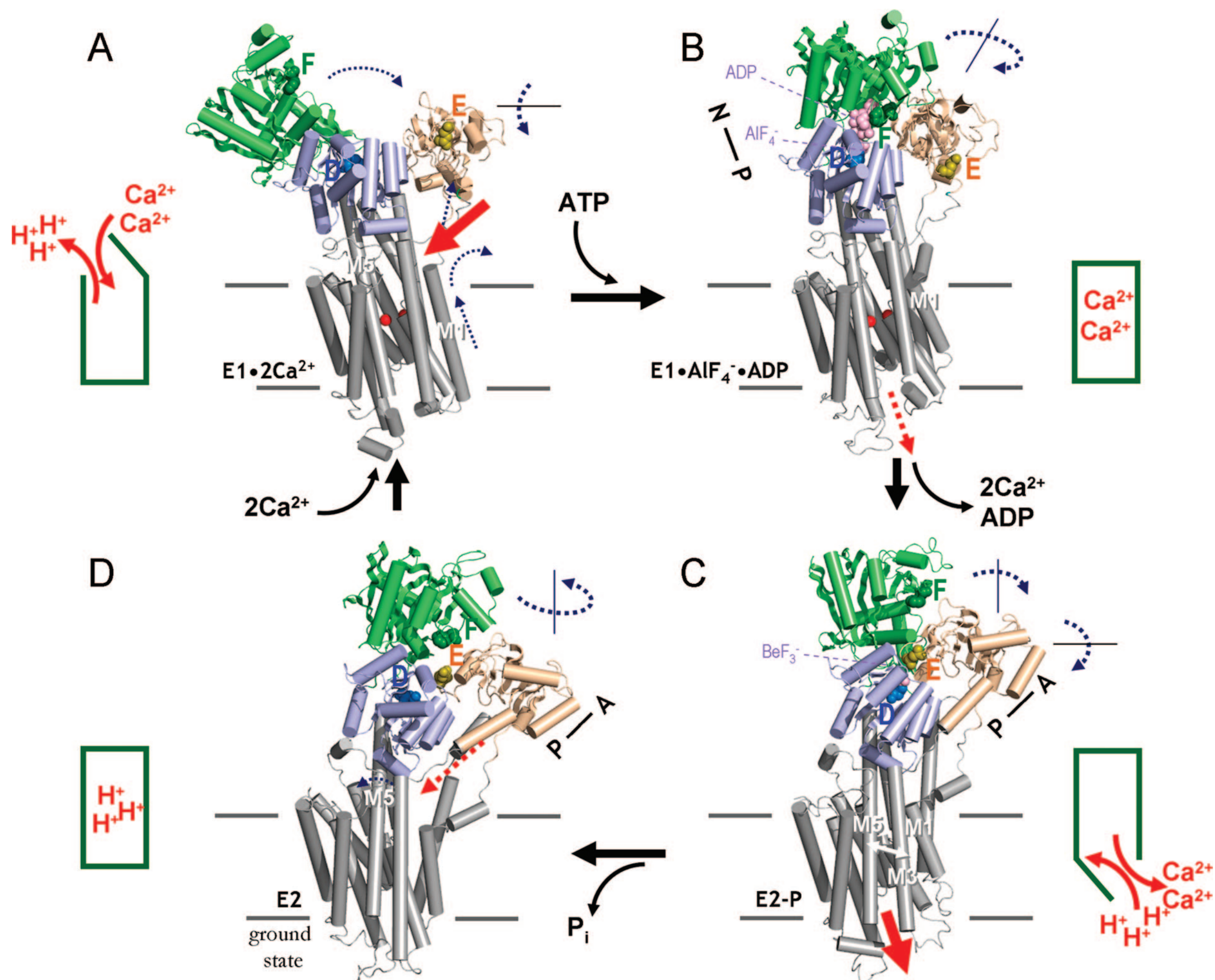

Figure 4. Conformational changes of SERCA1a during the catalytic cycle of Ca^{2+} transport. Four principle structures (A−D) depict the main events of Ca^{2+} transport. Green rectangles represent the sequential opening and closure of the cytoplasmic and luminal gates in the transmembrane region. (A) E1·2Ca^{2+} (1SU4[7]): Ca^{2+} entry and binding. The cytoplasmic gate is open, allowing the exchange of 2−3 H^+ for 2 Ca^{2+} ions. The putative ion-entry pathway is marked by the red arrow. (B) E1·2Ca^{2+}·ATP (represented by E1·2Ca^{2+}·AlF_4^-·ADP, 2ZBD[23]): phosphorylation and occlusion of the pump. ATP links the N- and P-domains. The pump becomes phosphorylated, locking the access to the Ca^{2+} sites. Note the lifted and kinked M1 helix that closes the entry site. (C) E2-P (represented by E2·BeF_4^-, 3B9B[27]): Ca^{2+} release, exchange for H^+. Reorientation of the A-domain opens the luminal exit pathway for Ca^{2+} (indicated by red arrow). The TGE-loop replaces ADP. (D) Ground-state E2 (represented by E2(TG), 2AGV[21]): dephosphorylation and occlusion of protons. Red arrows indicate the Ca^{2+}-entry (D and A) and -exit (B and C) pathways. Dashed lines indicate events that need to take place to reach the next step in the cycle. The A-domain is shown in orange, the P-domain in light blue, the N-domain in green, and the M-domain in gray. Ca^{2+} ions are shown as red spheres. D (dark blue) is Asp351, the phosphorylation site; F (dark green) is Phe487 of the ATP-binding site; E (orange) is Glu183 of the dephosphorylation motif. N−P, association of the N- and P-domains; P−A, association of the P- and A-domains (based on ref 38).

contributes to the binding of both Ca^{2+} ions.[7] The position of the oxygen atoms in site II resembles the structure of an EF-hand motif,[38] which is found in many other Ca^{2+}-binding proteins.[50]

The binding of the two Ca^{2+} ions is sequential and cooperative.[51] Ca^{2+} first meets the gating residue Glu309, which is part of site II but is not trapped because site II is not yet properly formed.[52] Instead, the first Ca^{2+} proceeds to site I, where it fits better. The binding of Ca^{2+} to Asp800 in site I induces a slight rotation of M6.[21,38] This will increase the Ca^{2+} affinity of binding site II,[48,53] which now can bind the second Ca^{2+}. Although Glu309 will capture the second Ca^{2+} ion in site II, this cytoplasmic gate can still open[38] and the Ca^{2+} ions remain under constant attack by water molecules and can be exchanged with other Ca^{2+} ions.

Phosphorylation and Occlusion: E1·2Ca^{2+} → E1∼P·2Ca^{2+} (Figure 4 A→B). The conformational changes following the induced fit of the two Ca^{2+} ions in the transmembrane region are transmitted to the cytoplasmic domains, 50 Å away, providing the signal allowing phosphorylation. This ensures that ATP hydrolysis only takes place when ions are loaded, so that a tight coupling between ion transport and energy expenditure is obtained.[54]

The phosphorylation process starts with binding of the adenosine of ATP to residue Phe487 of the N-domain. The γ-phosphate of ATP and a Mg^{2+} ion bridge the N-domain to the P-domain at residue Asp351 (N−P-link, Figure 4B).[22,26] During the transition toward the E1∼P phosphorylated state (presented by the E1·ADP·AlF_4^- structure[26]), the P-domain bends and brings Asp703 closer to the phosphorylation site to catalyze the phosphorylation reaction. The ATP molecule is now positioned for an S_N2 nucleophilic reaction,[22,26] which hydrolyses ATP and phosphorylates Asp351. The bending of the P-domain tilts the A-domain that rests on the P-domain, exerting strain on the link between the A-domain and M3. At the same time, M1−M2 is partially

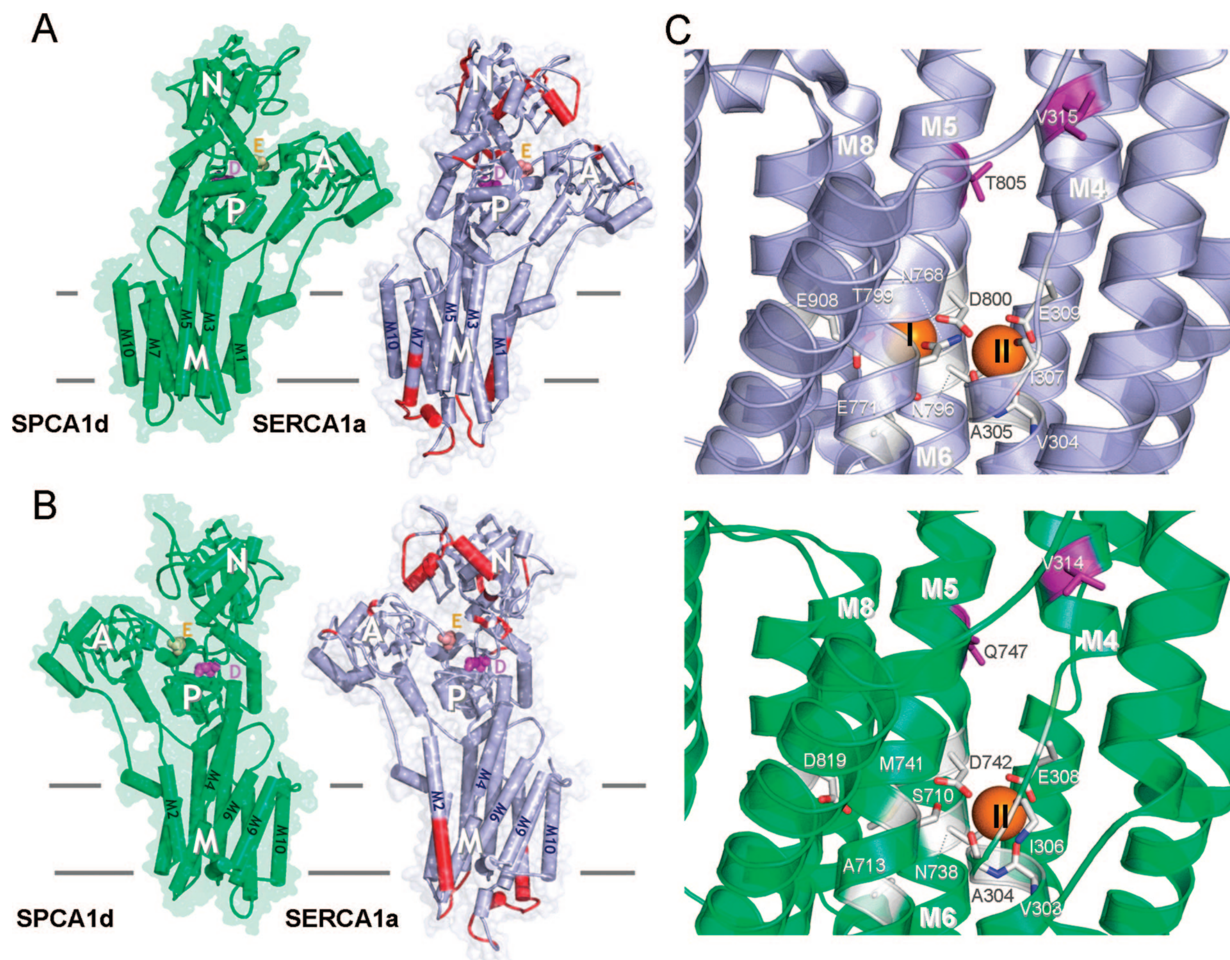

Figure 5. Homology model of SPCA1d. (A) Front view and (B) back view of the SPCA1d homology model calculated using MODELER[178] (green). The SERCA1a E2(TG) structure (2AGV)[30] was used as a template and is shown for comparison (blue). The longer N- and C-termini of SPCA1d were left out of the calculation. The longer loops in SERCA are indicated in red and are predominantly found in the N-domain and in the luminal loops. (C) Detailed view of the Ca^{2+}-binding site in E1·2Ca^{2+}: SERCA (top, 1SU4 structure[7]) and SPCA (bottom, E1 homology model). Only site II is perfectly conserved between SPCA and SERCA. The residues important for Mn^{2+} selectivity in SPCA (and the corresponding residues in SERCA) are indicated in purple. The Ca^{2+} ions are shown in orange. Oxygen (red), nitrogen (blue), and sulfur (yellow) atoms are colored.

lifted out of the membrane and the amphipathic N-terminal region of M1 forms a kink and bends over to lie flat on the membrane layer.[22,26] This closes the cytosolic access path to the binding sites and forces residue Glu309 in a fixed position.[22,38,44] Because the Ca^{2+}-entry pathway to the lumen is not yet open, the pump is now in an occluded state, preventing further exchange of Ca^{2+} ions from the cytosol.[22,26,55] This ensures a highly efficient separation between the cytoplasm and the luminal environment that is required for perfect coupling of Ca^{2+} transport and ATP hydrolysis.

Ca^{2+} Release: E1~P → E2-P (Figure 4 B→C). Once the ATP molecule forms a cross-link between the N- and P-domains, it holds the pump in a highly strained state. Upon phosphorylation, hydrolysis of ATP will break this tight association, and the N-domain carrying the ADP will dissociate from the P-domain.[22,27,38] This also relaxes the strained state and brings about a new rotation of the A-domain, switching the pump to E2-P. Here, the space close to the aspartyl phosphate is no longer occupied by ADP but rather by the TGE-loop of the A-domain.[27] This shields the phosphorylation site by restricting the access of ADP or water. Together with the massive rotation of the A-domain, transmembrane helices M1−M6 are rearranged and a luminal exit pathway for Ca^{2+} is formed by spreading out M1/M2 and M3/M4 away from M5/M6.[27,47] Rotation of M6 (Asp800) and a large downward movement of M4 (Glu309) distort the Ca^{2+}-binding sites and reduce the Ca^{2+} affinity, which allows a quick release of Ca^{2+} even in the high-Ca^{2+} background of the ER lumen.[21,27] Residues of the empty Ca^{2+}-binding sites are immediately stabilized by protons and water molecules.[30] In fact, protons for countertransport should be in place to allow hydrolysis of the phosphoenzyme. The position of the countertransport protons in SERCA is not resolved yet, but the residues Glu309, Glu771, and Asn796 are exposed to the luminal exit pathway and are likely candidates for proton binding.[27,49] In the Na^+,K^+-ATPase, the Rb^+ counterions (as K^+ congeners) interact with homologous residues (Glu327, Glu779, and Asp804).[42]

Dephosphorylation and Occlusion: E2-P → E2·P (Figure 4 C→D). The dephosphorylation reaction starts with the entrance of one water molecule into the phosphorylation site.[28] A new rotation of the A-domain is evoked, which accurately positions Glu183 of the TGE-loop to fix a water molecule and to catalyze its attack on the aspartyl phosphate via an associative S_N2 dephosphorylation reaction.[23,25,56] The rotation of the A-domain also affects the luminal gate by repositioning M1/M2 back in the membrane. M1/M2 and M3/M4 close against M5−M10, and the cation-binding site becomes occluded.[27] Thus, dephosphorylation of the Ca^{2+}-ATPase locks the luminal gate for protons.[23,25,27]

In the ground state E2, the ATPase retains a compact conformation.[21] Thermal agitation will open the headpiece to reach the more open E1 and E1·2Ca^{2+} state and to release the bound protons.[57] Jensen et al.[29] proposed an alternative mechanism involving smaller conformational changes. Accordingly, Ca^{2+} activation of the phosphorylation reaction can proceed directly from the compact Ca^{2+}-free E2-ATP state to another relatively compact Ca^{2+}-bound E1-ATP form. This is supported by the observation that E1 crystals for electron microscopy obtained in milder conditions appear to be more compact.[58] Crystal structures of the pump in E2 with bound AMPPCP (an ATP homologue) demonstrate that ATP can bind in a compact E2 conformation.[29] Given the millimolar concentrations of ATP in the cell, it is thus possible that the nucleotide interacts with E2[29,59] even before binding of Ca^{2+}. This would favor a compact conformation of the pump in E1, speeding up the rate of the subsequent phosphorylation. The observed overall stimulatory effect of Mg^{2+}-ATP on the rate of Ca^{2+} transport[60] would support this hypothesis.

Concluding Remarks on the Pumping Mechanism. Conformational changes in the cytosolic domains affect the relative position of the transmembrane helices, controlling the access to and affinity of the ion-binding sites. The Ca^{2+} pump effectively exploits inherently stochastic thermal motions to drive substantial conformational changes. The random thermal vibrations are effectively converted into a unidirectional series of domain rearrangements by suppressing the backward reactions.[38] Binding of the Mg^{2+}, Ca^{2+}, H_2O, and ATP ligands makes the energy barriers between the principal intermediates comparable to the thermal energy.[38] During the phosphorylation and dephosphorylation events, thermal fluctuations should be minimized to obtain the precise linear arrangement for the associative reaction mechanism. A vast number of ligand–domain, interdomain, and intradomain interactions temporarily forces the pump in a locked state.[38,44]

3.3. SERCA Proteins

3.3.1. SERCA1

SERCA1 was the first described isoform of the SERCA family. Its expression is almost entirely confined to fast-twitch skeletal-muscle fibers, where it represents the most abundant intrinsic membrane protein in the sarcoplasmic reticulum (SR). Mutations in the *ATP2A1* gene encoding SERCA1 in fast-twitch skeletal muscle cause Brody myopathy (OMIM 601003, Box 1), an autosomal recessive inherited disease with exercise-induced impairment of fast-twitch muscle relaxation in humans due to slow Ca^{2+} removal from the myoplasm.[61] Several animal models for Brody disease showing mutations in the *ATP2A1* gene have been described. Among these are congenital pseudomyotonia in Chianina cattle, which is due to a missense mutation in the actuator domain,[62] and congenital muscular dystonia 1 in Belgian Blue cattle, which is caused by a mutation in the ATP-binding site.[63] Zebrafish behavioral mutants known as *accordion* also represent Brody models. They show strongly impaired tail-muscle relaxations and carry missense mutations in one of the three transmembrane segments (M2, M5, or M7) of the *ATP2A1* gene.[64] In contrast to the situation in human and in Chianina cattle where the absence of functional SERCA1 appears to be tolerated reasonably well, affected Belgian Blue calves usually die within a few weeks as a result of respiratory complications. The situation is even more severe in homozygous *ATP2A1* knockout mice, which do not survive birth due to failure of the diaphragm to support respiration.[65]

All SERCA genes, from fish to mammals, are expressed as different isoforms as a result of alternative splicing of the gene's primary transcript.[20] In the case of SERCA1, this splicing is developmentally regulated and results in a neonatal SERCA1b and an adult SERCA1a isoform.[66] In the neonatal isoform, a highly conserved octapeptide (–DPEDERRK) stretch consisting of mostly charged amino-acid residues replaces the C-terminal Gly residue found in the adult isoform. The physiological meaning of this neonatal isoform remains at present unknown.[66] The adult isoform results from the inclusion in the mature messenger of an optional exon (exon 22) that is spliced out in the neonatal condition. The retention of exon 22 in the adult SERCA1 mRNA is critically dependent on the presence of an alternative splice factor called *muscle blind like*, which binds to a series of CUG motives found in the intron immediately downstream of exon 22 in the *ATP2A1* transcript to promote the retention of this exon. In type-1 myotonic dystrophy (OMIM 160900), the muscle blind like protein is titrated away by binding to an excessively long pathogenic $(CUG)_n$ microsatellite expansion in the 5′-UTR of the DMPK (dystrophia myotonica protein kinase). The thus-created relative shortage of muscle blind like not only results in the expression of the neonatal SERCA1b in muscle but also causes a trans-dominant disturbance of the alternative splicing in a whole series of other gene transcripts including the ryanodine receptor (RyR), a chloride channel and an insulin receptor.[67]

3.3.2. SERCA2

Properties and Expression. Of the three mammalian members belonging to the SERCA family, SERCA2 is evolutionary the oldest and orthologous to the invertebrate SERCA protein. It is remarkable that alternative processing of this SERCA-gene transcript, both in the invertebrates as well as in the vertebrates, can lead to at least two major different protein variants corresponding to the human SERCA2a and SERCA2b. The difference between the a isoform and the b isoform lies in the C-terminus. The b form of the invertebrate SERCA and of the vertebrate SERCA2 shows a C-terminal tail that is always a few tens of amino-acid residues longer than the a isoform, and it contains a segment with the propensity of forming an additional 11th transmembrane helix.[68–70] In vertebrates, SERCA2b is the housekeeping variant and SERCA2a is a muscle-specific variant (expressed in the heart, slow-twitch skeletal muscle, and smooth-muscle cells).[71] The last 4 amino acids of SERCA2a are replaced by a longer extension of 49 residues in SERCA2b, which contains an 11th transmembrane segment.[72] The C-terminus of SERCA2b therefore resides in the lumen of the ER.[72,73] Both in *Caenorhabditis elegans*[68] and in vertebrates,[20,74,75] the extended tail in the b variant increases the affinity for cytosolic Ca^{2+} and lowers the maximal catalytic turnover rate compared to the shorter a form. It was shown for the human SERCA2b that its longer tail compared to SERCA2a mediates a reduction of the rate of Ca^{2+} dissociation from the Ca^{2+}-binding sites and slows the E1∼P to E2-P and E2-P to E2 conversions. These changes in the kinetics of SERCA2b explain the higher apparent affinity for Ca^{2+} and the lower maximal turnover rate.[76]

Recent PCR analysis pointed to the presence of mRNA encoding a third human variant, SERCA2c, in human monocytes and in cancers of myeloid and epithelial origin.[77] However, it appears that its expression level relative to SERCA2b must be low since it required an extended number of PCR cycles to amplify the corresponding SERCA2c fragment, and there is only one reported SERCA2c-related EST fragment in the databases (May 2009). This variant was expressed in the cardiac left ventricle, where again it only made up a small fraction of the total SERCA content.[78] SERCA2c contains a unique C-terminus of 6 amino acids, which lowers the Ca^{2+} affinity of the pump with respect to SERCA2a.[78]

SERCA2 Mouse Models. The role of SERCA2a in cardiac excitation−contraction coupling is well-understood (reviewed in refs 79−81). In brief, SERCA2a activity is a major determinant of the kinetics and force of cardiac contraction and relaxation since it controls the rate of removing cytosolic Ca^{2+}, the SR Ca^{2+} load, and therefore also the amount of Ca^{2+} available for contraction.[82] Transgenic overexpression of SERCA1a[83] or SERCA2a[84] improved the speed of cardiac relaxation and contraction. The opposite is true for heterozygous knock-down of SERCA2.[85] Homozygous *SERCA2*$^{-/-}$ mice are embryonically lethal.[85]

A reduced SERCA2a activity would at least partially reflect the diminished cardiac contractility in heart failure.[86,87] Enhancing the activity of SERCA2a therefore seems an appealing strategy to reverse the progression of heart failure. In the past decade, it has been repeatedly demonstrated in several animal models of this disease that increased SERCA2a activity translates in better contractile properties and sometimes reverses the disease's progression (reviewed in refs 79−81). Currently, a clinical phase-I trial is ongoing for the treatment of heart-failure patients via adenoviral delivery of SERCA-gene copies in the heart.[88]

Contrary to expectations, SERCA2 haploinsufficiency in mice[85] and humans[89,90] is not associated with cardiac disease, but is manifested in the skin. Heterozygous *SERCA2*$^{+/-}$ mice are prone to develop squamous cell tumors,[91] whereas humans lacking a functional copy of SERCA2 are affected by Darier disease (OMIM 124200, Box 1),[92] a skin disease characterized by a disruption of cell−cell contacts (acantholeysis) in the suprabasal layers of keratinocytes in the epidermis.

Studies in our laboratory addressed the physiological meaning of the SERCA2a/b diversity in the heart. In the *SERCA2*$^{b/b}$ mouse model, the SERCA2a isoform was substituted by the higher Ca^{2+}-affinity variant SERCA2b. The adult *SERCA2*$^{b/b}$ animals developed concentric left-ventricular cardiac hypertrophy with impaired cardiac contraction and relaxation, showing that it is specifically the SERCA2a isoform that is essential for normal cardiac growth and function.[93]

In the *SERCA2*$^{b/b}$ mouse model, the high Ca^{2+} affinity of SERCA2b was at least partially offset by the affinity-modulator phospholamban (PLN, see next paragraph), which played a protective role.[94] In addition, the SERCA-expression level in the heart of *SERCA2*$^{b/b}$ mice was halved,[93] which might also help to offset the increased SERCA activity in the low cytosolic Ca^{2+}-concentration range by the higher affinity variant SERCA2b.[94,95] Whereas in *SERCA2*$^{b/b}$ mice the partial loss of SERCA2b expression was accompanied by a stronger PLN inhibition,[94] the lower SERCA2a expression in two other mouse models (the heterozygous *SERCA2*$^{+/-}$ [96] and *SERCA2*$^{b/WT}$ mice[95]) was effectively compensated by a *reduced* inhibition by PLN. This suggests that, in the *SERCA2*$^{b/b}$ mice, compensation of the high Ca^{2+} affinity of SERCA2b by PLN is more important than maintaining normal SERCA2-expression levels, highlighting the importance of controlling the Ca^{2+} affinity of the cardiac SERCA pump.

Small Transmembrane Regulators PLN and Sarcolipin. PLN is by far the best-studied endogenous regulator of SERCA (extensively reviewed in refs 80, 81, and 97). PLN follows the expression pattern of SERCA2a, with the highest expression levels in the heart[98,99] and some expression in slow-twitch skeletal muscle[100] and smooth-muscle cells.[101] This 52-amino-acid long protein consists of a cytosolic and a transmembrane domain. PLN physically interacts with the SERCA pump[102] and inhibits Ca^{2+} transport by lowering the apparent Ca^{2+} affinity of the pump.[97] PLN inhibits the activity of SERCA1a, SERCA2a, and SERCA2b, but not of SERCA3.[75] Guided by mutagenesis and cross-linking results, the PLN NMR structure was modeled on the SERCA1a crystal structure. This modeling placed the transmembrane region of PLN in the hydrophobic groove formed by M2, M4, M6, and M9 in the E2 conformation of SERCA. As SERCA moves to the E1 conformation, M2 is displaced, which narrows this groove, making it unsuitable for PLN interaction.[103,104] PLN is therefore thought to hold the pump preferentially in the E2 conformation, consequently reducing the apparent affinity for Ca^{2+}. The cytosolic PLN domain interacts with residues in the N-domain of the pump.[102] Phosphorylation of PLN dissociates the functional interaction with the Ca^{2+} pump but is less effective than Ca^{2+} binding to SERCA in breaking up physical interactions.[105] PLN pentamers act as a reservoir for PLN monomers, the predominant active form, which can readily dissociate from the pentamer when dephosphorylated.[81] The extent of PLN phosphorylation thus critically determines its inhibitory properties. At least two sites are phosphorylated during β-adrenergic stimulation in vivo: Ser16 is phosphorylated by cAMP-dependent protein kinase and Thr17 by Ca^{2+}/calmodulin-dependent kinase II (CaMKII).[106]

In resting conditions, PLN is a physiological brake of SERCA2a Ca^{2+} transport that inhibits cardiac contractility.[79] During β-adrenergic stimulation, this inhibition is relieved by phosphorylation of PLN, which substantially contributes to the inotropic (enhanced contraction) and lusitropic (enhanced relaxation rate) effects of β-stimulation.[107] The role of the dual phosphorylation sites on PLN has been extensively investigated in transgenic mice expressing phosphorylation-site-specific mutants in the PLN null background.[108,109] These studies suggest that phosphorylation on Ser16 is sufficient to mediate a full β-adrenergic effect in vivo and that Ser16 phosphorylation is a prerequisite for phosphorylation on Thr17. However, the failure to find PLN phosphorylation in transgenic mice in which the Ser16 site was mutated to Ala can be attributed to the fact that the lack of phosphorylation of the Ser16 site precludes the increase in cytosolic [Ca^{2+}] necessary to activate CaMKII and phosphorylate Thr17. In fact, Thr17 phosphorylation, independent from Ser16, has been observed in several (patho)physiological conditions (reviewed in ref 110). Whereas Ser16 would be the predominant site of phosphorylation during β-adrenergic stimulation, Thr17 could be important to mediate the frequency-dependent acceleration of relaxation (FDAR). FDAR is impaired in the Thr17Ala PLN transgenic mice,

suggesting that the frequency-dependent increase in phosphorylation of Thr17 by CaMKII plays a major role in the positive force-frequency relationship in cardiomyocytes.[111] This view is, however, challenged by other observations, e.g., those showing that PLN is not required for mediation of FDAR.[112] The reader is further referred to other reviews that treat the physiological importance of PLN in cardiac-muscle contractility into more depth.[79−81,113]

Recently, sarcolipin (SLN) emerged as another regulator of the cardiac SERCA2a pump. Initially, SLN was considered as the PLN counterpart in skeletal muscle, regulating exclusively SERCA1a activity. Recent studies, however, demonstrated that SLN is coexpressed with SERCA2a and PLN in the atria of the heart (but not in the ventricles) and in slow-twitch skeletal muscle.[98,99] In vitro, SLN is able to lower the apparent Ca^{2+} affinity of SERCA1a and SERCA2a. SLN could also regulate SERCA2a activity in vivo, as shown by adenoviral overexpression of SLN in isolated cardiomyocytes[114] or transgenic SLN overexpression in the mouse heart.[115,116] SLN is a 31-amino-acid long PLN homologue. Compared to PLN, SLN has a much shorter cytosolic domain but a slightly longer, highly conserved luminal C-terminus (R^{27}SYQY). This extension is not only important for proper targeting and insertion into the ER membrane[117] but is also crucial for interacting with the luminal face of SERCA1a.[118] Like PLN, SLN directly interacts with the Ca^{2+} transporter, lowering its apparent affinity for Ca^{2+}. SLN presumably occupies the same hydrophobic binding groove in the E2 conformation (M2, M4, M6, M9) as PLN, suggesting a similar mode of action.[119,120] Moreover, this hydrophobic groove could accommodate both SLN and PLN simultaneously.[119,120] SLN and PLN enforce each other's function in vitro, synergizing as a super inhibitor of either SERCA1a or SERCA2a,[121] but it remains to be determined whether this superinhibition plays a physiological role.

In contrast to earlier reports,[122] more recent studies suggest that, as for PLN, the activity of SLN can be regulated by phosphorylation. This might explain why the inhibitory effect of overexpressed SLN on cardiac contractile function could be reversed by treatment with isoproterenol, a β-agonist,[115,116] even in the absence of endogenous PLN.[123] Conversely, loss of SLN in the atria was found to be associated with a blunted β-adrenergic response.[124] Together, these studies suggest that SLN, like PLN, could play a role in the β-adrenergic stimulation of the atria.[81] The exact mechanism by which SLN responds to adrenergic activation is yet to be determined. Predicted phosphorylation sites in SLN are Ser4 (cow, rabbit, pig SLN) or Thr5 (human, mouse, rat SLN). At least the serine−threonine protein kinase 16 is able to modulate SLN-dependent inhibition of SERCA1a activity in vitro,[123] but its physiological relevance remains unclear.[123]

Other Regulators of SERCA2. PLN and SLN are the best known endogenous regulators of SERCA2, but in the last few decades, several other SERCA-interacting proteins and post-translational modifications of SERCA2 that modulate the activity of this Ca^{2+} pump were identified. The vast literature concerning this topic was recently reviewed elsewhere[125] and will not be further treated in depth. Some of the proteins that were suggested to modulate SERCA2 activity are: the antiapoptotic protein Bcl-2,[126] the insulin-receptor substrates IRS1/2,[127] the EF-hand Ca^{2+}-binding protein S100A1,[128] acylphosphatase,[129] G-protein-coupled receptors,[130] TRPC1,[131] and HAX-1.[132] Possible interactions with SERCA2 in the ER lumen were described for calreticulin, calnexin, calmegin, and ERp57.[133] *N*-glycosylation, glutathionylation, nitration, and phosphorylation are among the post-translational modifications that have been suggested to modulate the activity of the pump. Maladaptations in several of these regulatory mechanisms are linked to disease (reviewed in ref 98).

3.3.3. SERCA3

Properties. SERCA3 differs from the other SERCA isoforms by its remarkably low affinity for cytosolic Ca^{2+} (supramicromolar) and luminal Ca^{2+} (lower millimolar),[134] its insensitivity to PLN,[135] and its higher resistance to oxidative damage.[136] The lower affinity for cytosolic Ca^{2+} can be partially ascribed to a reduction in the E2- to E1-transition rate. A longer dwelling time in the low-affinity E2 conformation results in a lower apparent affinity for Ca^{2+} and is accompanied by a higher sensitivity for vanadate.[134,136] Besides the kinetic effect, SERCA3 also manifests a true lower affinity for binding not only of cytosolic but also of luminal Ca^{2+}. The physiological relevance of the lower Ca^{2+} affinity of SERCA3 remains unknown, but a pump with a low sensitivity to luminal Ca^{2+} might be better suited to accumulate Ca^{2+} to higher levels in the ER.

Expression. Alternative processing of the transcripts of the SERCA3 gene results in at least 6 different isoforms in human (SERCA3a−f), 3 in mice (SERCA3a−c), and 2 in rats SERCA3a, b/c).[20,137] Like for SERCA1 and SERCA2, these SERCA3 isoforms also differ only in their C-terminus. It is too early to understand the physiological/pathological consequences of this bewildering isoform diversity. Some of these splice variants like, e.g., the human SERCA3d and SERCA3f were reported to be targeted to a specific subcellular localization in the cardiomyocyte or to cause ER stress when overexpressed (SERCA3f),[137] but as yet no clear expression/function-relation pattern can be discerned.

Factors controlling SERCA3 expression are complex and poorly understood, but SERCA3 can be used as a differentiation marker in vascular endothelial cells[138] and in colon mucosa.[139] In both cell types, SERCA3 expression decreases when cells dedifferentiate and proliferate. The loss of SERCA3 expression is an early event accompanying colon and stomach carcinogenesis and is controlled by the APC/β-catenin/TCF4 pathway.[139]

As for many genes with TATA-less promoters, the 5′-flanking region of the human SERCA3 gene (*ATP2A3*) is embedded in a CpG-island characterized by the presence of multiple Sp1 (specificity protein 1) sites as well as a single Ets-1 (E twenty-six 1) site.[140] The basal transcription of the mouse SERCA3 gene was shown to be governed by Ets-1 and Sp1.[141] Also the Ca^{2+}-dependent activation of SERCA3 transcription, which is regulated by the calcineurin/NFAT (nuclear factor of activated T-cells) pathway in endothelial cells, makes use of Ets-1 activation.[142]

SERCA3 Mouse Models. While *SERCA1*$^{-/-}$ mice develop respiratory failure shortly after birth[65] and SERCA2 ablation is embryonically lethal,[85] SERCA3 ablation is well-tolerated and not accompanied by an overt disease phenotype.[143] In view of the high expression of SERCA3 in pancreatic beta cells, and reports linking mutations in the human *ATP2A3* gene encoding SERCA3 with diabetes mellitus type 2,[144] Arredouani et al.[145] investigated the effects of SERCA3 ablation in mice. Glucose-induced cytosolic Ca^{2+} oscillations were only mildly affected, probably because Ca^{2+} uptake into the ER is largely mediated by SERCA2b at near-

resting Ca^{2+} levels. SERCA3 became active only at higher cytosolic [Ca^{2+}], where it blunted the Ca^{2+} oscillations. The loss of SERCA3 had no major impact on insulin secretion, and the *SERCA3*$^{-/-}$ mice did not develop diabetes.[145]

Differentiated vascular endothelial cells express high levels of SERCA3.[138] Here too, in spite of reported reductions in acetylcholine-induced endothelium-dependent relaxation of aortas precontracted with phenylephrine,[143] blood pressure is normal in homozygous SERCA3 null mutants.[145]

3.4. SERCA Inhibitors

The transition-metal oxoanion vanadate (VO_4^{3-}) is a characteristic inhibitor of P-type ATPases and is believed to mimic the transitional state of the phosphoryl group in the E2 conformation of the enzyme. Vanadate inhibits all P-type ATPases and will therefore not be discussed further. We will focus on selective high-affinity inhibitors of SERCA pumps: thapsigargin, cyclopiazonic acid, 2,5-di-*tert*-butyl hydroquinone, and artemisinins.

3.4.1. Thapsigargin

Thapsigargin, a plant-derived sesquiterpene lactone extracted from *Thapsia garganica*,[146] is the most specific known inhibitor of SERCAs. Thapsigargin binds with subnanomolar affinity[147] to SERCA1 in a wedge-shaped space bordered by the M3, M5, and M7 transmembrane helices.[21,148] It stabilizes the pump by restricting the movement of the helices relative to each other. This efficiently holds the pump in a Ca^{2+}-free E2-like conformation, with a minimal effect on the structure.[27,148] Although all mammalian SERCA isoforms bind thapsigargin with high affinity, the sensitivity may slightly differ, with SERCA1 being most sensitive and SERCA3 showing the lowest affinity.[147] Mammalian SERCA isoforms can become less sensitive to thapsigargin after prolonged treatment of cultured cells in the presence of the inhibitor.[149]

The binding is specific for the mammalian SERCA1−3, with much less and often no inhibitory effect on other P-type ATPases like PMCAs and SPCAs. One notable exception appears to be the AtHMA1 heavy-metal pump in *A. thaliana*, which belongs to the P_{1B}-ATPase subfamily and which can also transport Ca^{2+}. This is the first described plant P-type pump that is specifically inhibited by thapsigargin.[150] In contrast, the plant ECAs that are related to the mammalian SERCA pumps[151] and the SERCA orthologue PfATP4 in *P. falciparum*[152] are insensitive to thapsigargin.

3.4.2. Cyclopiazonic Acid and 2,5-Di-tert-butyl Hydroquinone

Cyclopiazonic acid is an indole tetramic acid fungal toxin. 2,5-Di-*tert*-butyl hydroquinone is a synthetic compound that is sometimes used as an antioxidant. They bind with a much lower affinity than thapsigargin to the SERCA. Their binding site in the transmembrane domain of the ATPase is clearly distinct from the thapsigargin-interaction site and is located closer to the cytosol. Indeed, SERCA1 crystals where thapsigargin and 2,5-di-*tert*-butyl hydroquinone were simultaneously bound have been obtained.[24,148] The binding domains for cyclopiazonic acid and 2,5-di-*tert*-butyl hydroquinone partially overlap and are found in what is believed to be the cytosolic Ca^{2+}-access channel of the ATPase. They block the ATPase in an E2-like state by locking M1 and M2 against M4. The inhibition can be reversed by raising the [ATP] and [Ca^{2+}].[24] Interestingly, cyclopiazonic acid and 2,5-di-*tert*-butyl hydroquinone also inhibit the SERCA orthologues in plants and *P. falciparum*.[151]

3.4.3. Artemisinin

Artemisinin is a sesquiterpene lactone endoperoxide extracted from the sweet wormwood (*Artemisia annua*) and a potent antimalarial agent that kills the malaria parasite. The SERCA orthologues of *P. falciparum*[153] and *Toxoplasma gondii*,[154] but not the vertebrate SERCAs, are inhibited by artemisinin, resulting in a perturbed Ca^{2+} homeostasis in these species.[154] Although the peroxide action on a variety of different targets in the parasite might explain the antimalarial action of the artemisinins, there is strong support for the view that the binding of artemisinin to the SERCA of the parasite might certainly contribute to its action. The reader is referred to a recent review by Krishna et al.[155] for further details. The binding of artemisinin or of its derivatives dihydroartemisinin, artmether, arteether, and artesunate[156] is thought to occur overlapping with, or close to, the thapsigargin-binding site, i.e., at the transmembrane domain in a cleft bordered by M3, M5, and M7. Indeed, single amino-acid substitutions in M3 could abolish artemisinin inhibition of pfATPase6.[157] However, the exact binding geometry of artemisinin remains to be further specified. Unfortunately, the sequences of the thapsigargin-binding pocket of SERCA1a, which is known from various crystal structures, and of the corresponding segments of the *Plasmodium* pump are too divergent to allow detailed modeling.

4. SPCAs

The SPCAs are, together with the SERCAs, responsible for loading the nonmitochondrial intracellular stores with Ca^{2+}. They both belong to the P_{2A}-ATPase phylogenetic subgroup. The characteristics of the SPCAs have been covered in comprehensive reviews.[20,158,159] Here, we will mainly focus on the more recent findings.

4.1. Genes Encoding SPCAs

The first member of the SPCA family was discovered and initially characterized in the yeast *S. cerevisiae* and designated Pmr1 (for plasma membrane ATPase-related). The *PMR1* gene was independently identified in the mid 1980s by two laboratories: Smith et al.[160] cloned the gene by complementation of "supersecreting" yeast mutants (*ssc*) that secrete large amounts of heterologously expressed proteins, while Serrano et al.[161] identified the same gene by its homology to the *PMA1* gene for the yeast plasma-membrane H^+-ATPase via low-stringency hybridization with a *PMA1* probe. Later on, homologues were discovered in multicellular model systems like *Homo sapiens*,[162−164] *C. elegans*,[165] and *Drosophila melanogaster*,[166] but also in a number of other fungi like *Kluyveromyces lactis*,[167] *Yarrowia lipolytica*,[168] *Hansenula polymorpha*,[169] *Schizosaccharomyces pombe*,[170] *Aspergillus niger*,[171] *Candida albicans*,[172] and *Pichia pastoris*.[173]

In humans, the *ATP2C1* gene encoding SPCA1 was mapped to chromosome 3 and proved to be the gene defective in the Hailey−Hailey human skin disease (OMIM 169600, Box 1).[162,163] Although there was some initial confusion about the alternative splicing of the gene product,

the unifying study of Fairclough et al. eventually described four different splice variants.[174] The resulting proteins are termed SPCA1a–d and only differ in their C-termini. The presence of a second member of the SPCA family was implicitly suggested by Ishikawa et al.[175] from a predictive screen for cDNAs encoding large proteins in the brain. In 2005, the human *ATP2C2* gene product (SPCA2) was simultaneously characterized by Vanoevelen et al.[176] and Xiang et al.[177]

4.2. Structure of SPCAs

The published structures of SERCA1a serve as excellent templates for homology modeling of related P-type ATPases. Homology models were previously described for several P-type ATPases, including Na^+,K^+-ATPase,[178,179] H^+-ATPase,[180] PMCA,[181] and H^+,K^+-ATPase.[182] Here, we present two homology models of the human SPCA1d based on the SERCA1a structures E1·$2Ca^{2+}$ (1SU4) and E2 (1IWO) as templates (Figure 5, calculated using MODELLER[178]). This pump displays a 29% sequence identity and 43% similarity with the rabbit SERCA1a. The longer N- and C-termini of SPCA were excluded from the model due to the lack of additional structural information.

In general, the SPCA sequence is shorter and more compact than SERCA (parts A and B of Figure 5). The SERCA protein displays longer cytosolic and luminal loops, possibly containing unique regulatory and interaction sites. At least some of these longer loops in SERCA are known protein-interaction sites: binding of ERp57 to SERCA's luminal loop L7–8 modulates the redox state of the pump,[133] and interaction of HAX-1 with the longer cytosolic loop 575–594 of the SERCA2 N-domain regulates the protein levels of the pump.[132] In the next sections, some structural features of the SPCA pumps are highlighted.

4.2.1. Mn^{2+} and Ca^{2+} Binding

In contrast to the SERCA Ca^{2+} pumps, the SPCA pumps, like the PMCAs, transport only 1 Ca^{2+} ion per ATP. This would be consistent with the presence of only one Ca^{2+}-binding site. Indeed, only the side chains contributing to the Ca^{2+}-binding site II are perfectly conserved between SERCA and SPCA (Figures 1 and 5C). The SPCA1-E1-homology model predicts a similar orientation of the side chains contributing to Ca^{2+} binding in site II. Site I is not suited to accommodate a Ca^{2+} ion in SPCA. Site I confers no (in PMCA) or only one shorter (in SPCA) acidic residue. Also the size and shape of the site-I cavity are different: the bulkier Met741 in SPCA replaces Thr799, and some of the side chains are smaller (Figures 1 and 5C).

All Ca^{2+}-ATPases thus have Ca^{2+}-binding site II in common, suggesting that site II is more fundamental to the operation of the Ca^{2+} transporters[45] (Figure 5C). Site II contains the gating residue (Glu309 in SERCA1), which is not only important for Ca^{2+} binding but is also crucial for the subsequent phosphorylation and occlusion of Ca^{2+} ions.[52,183] This conserved ion-binding site in SPCA supports the view that similar Ca^{2+}-binding mechanisms apply to both pumps, with Glu308 (the homologue of Glu309 in SERCA1) likely functioning as the gating residue.

Three interesting features of SPCAs suggest that ion selection and entry in these transporters may be fundamentally different from SERCA1a transporters. *First* is the transport of Mn^{2+}, a particular characteristic of SPCA pumps. Other Ca^{2+}-ATPases display very weak affinity toward Mn^{2+} and transport Mn^{2+} only at high, nonphysiological Mn^{2+} concentrations. In SPCAs, the transport of Mn^{2+} and Ca^{2+} is mutually exclusive, suggesting that both ions occupy the same ion-binding site.[165,184] This implies that discrete structural elements provide the Mn^{2+} selectivity of SPCA pumps. At least some residues that are crucial for Mn^{2+} selectivity were identified in the yeast Pmr1. These residues are located at the cytoplasmic side of the ion-binding site, between the side chains of Gln783 (M6) and Val335 (M4),[185,186] and they are conserved in the human SPCA1 (the homologues Gln747 and Val315 are shown in Figure 5C) and SPCA2 isoforms. This region may constitute an access gate for Mn^{2+} ions. Gln783 in M6 may form a critical hydrophobic interaction with Val335 in M4, which is important for correct packing of the transmembrane helices.[185] *Second*, the N-termini of PMR1 and SPCA1 contain an unpaired EF-hand-like motif, which is a helix–loop–helix structure (not shown in the models of Figure 5). In the yeast Pmr1, this motif binds Ca^{2+} and modulates the ion transport.[187] *Third*, the negatively charged residues in the W[50]ELVIEQFEDLLVRI sequence would shape the putative Ca^{2+}-entry site in M1 of SERCA1a. At least some of these residues were shown to participate in the binding of a divalent metal ion (Mn^{2+} or Mg^{2+}) in the presence of cyclopiazonic acid, which may mimic the binding of Ca^{2+} along the entry pathway.[188] Importantly, these residues are highly conserved in SERCAs and PMCAs (L[94]ELVWEALQDVTLII in hPMCA4b)[44] but are completely lacking and replaced by positive charges in SPCA (W[72]**KK**YISQF**K**NPLIML in hSPCA1d), pointing to fundamental differences in the entry pathway of these pumps.

4.2.2. Countertransport

The traditional view is that all P-type ion-motive ATPases catalyze an exchange of ions. After having translocated ions from the cytosol to an extra-cytosolic compartment, they transport other ions from the extra-cytosolic compartment to the cytosol (K^+ for Na^+,K^+-ATPase and H^+,K^+-ATPase, H^+ for SERCA and PMCA). The pronounced leakiness of the ER membrane for protons[189] questions the physiological relevance of proton countertransport in the SERCAs. It has, however, been demonstrated that proton countertransport serves a structural role and is required for stabilizing the empty Ca^{2+}-binding pocket in the E2 conformation.[30] Thus, countertransport seems fundamental to the operation of SERCA1a, and it has been suggested that it could be mandatory to all P-type ATPases.[190] It is well-known that the addition of K^+ ions or lowering the pH stimulate the dephosphorylation reaction (E2-P → E2) of SERCA.[191,192] However, SPCA resides in the Golgi membrane, which is much less proton-permeable than the ER. The dephosphorylation reaction of SPCAs is constitutively highly activated and is not further stimulated by protons or K^+ ions, which may imply that SPCAs do not perform countertransport.[193] This could be an important adaptation of SPCA pumps to prevent protons from leaving the acidic Golgi compartment. In the Golgi, V-type ATPases actively accumulate protons and the tight Golgi membrane prevents protons from leaking out. Preventing proton countertransport in SPCA pumps would contribute to maintain a low pH in the Golgi apparatus, which is essential to support several vital functions.[194] Compensation of the empty binding site in SPCA may not be crucial, since after the release of only one Ca^{2+}, at least fewer negative charges are present in SPCA than in

SERCA. Even in SERCA pumps, not all negative charges in the E2 form are fully compensated by counterions.[30,34,35] Also plant proton pumps do not exhibit countertransport, further questioning whether this really is a universal feature of P-type ion transporters. Here, a built-in counterion (Arg655) neutralizes the deprotonated negatively charged aspartate (Asp684) in the E2 conformation.[41,195]

4.3. Expression of SPCAs

4.3.1. SPCA1

Cell-Specific Expression. SPCA1 is believed to be the housekeeping Ca^{2+} and Mn^{2+} pump of the secretory pathway because it is expressed in all cells. However, different laboratories have obtained slightly different results on the relative expression level in various tissues. Wootton et al.[196] observed much higher mRNA and protein levels in rat brain and testis than in other tissues like lung and liver. Such relatively high SPCA-mRNA expression, when compared to other tissues, was not observed in human brain and testis.[163,176] The cell-type dependent expression of SPCA1 in various animal species thus merits further study.

Subcellular Localization. The yeast PMR1 is localized in the Golgi apparatus or one of its subcompartments.[197] SPCA from *C. elegans* heterologously expressed in COS-1 cells[165] and the human SPCA1 expressed in CHO cells[198] also appeared in the Golgi area. It is now well-established that also the endogenous SPCAs in a whole range of cells are present in the Golgi compartment (reviewed in ref 199).

SPCA1 in human spermatozoa seems to occupy an unusual localization: it is found in an area behind the nucleus extending into the midpiece. SPCA1 may be the only intracellular Ca^{2+} pump in these cells because both functional and immunocytochemical tests failed to demonstrate the presence of SERCAs.[200] Thapsigargin concentrations up to 1 μM did not influence cytosolic [Ca^{2+}] in sperm cells. Higher concentrations of thapsigargin and bisphenol disturbed intracellular Ca^{2+} homeostasis, with an elevation of the resting cytosolic [Ca^{2+}] and an inhibition of the normal Ca^{2+} oscillations. Intracellular Ca^{2+} stores in sperm cells may therefore rely on a thapsigargin-insensitive Ca^{2+} pump different from the SERCAs of normal somatic cells. This pump is probably SPCA1. Sea-urchin (*Strongylocentrotus purpuratus*) sperm cells also appear to lack SERCAs. Their SPCAs are located in the zone occupied by the single giant mitochondrion where the main ATPases involved in Ca^{2+}-store filling are situated.[201,202]

In *D. melanogaster*, the SPCA-homologue SPoCk is alternatively spliced in three products termed SPoCk-A, SPoCk-B, and SPoCk-C. Interestingly, only the SPoCk-A isoform is localized in the Golgi apparatus. The localization of SPoCk-B and C is less clear, and unexpected targeting to, respectively, the ER and the peroxisomes was reported.[166] In this context, it should be mentioned that peroxisomes of mammalian cells appear to lack active Ca^{2+}-uptake systems altogether.[203]

4.3.2. SPCA2

Analysis of the databases indicates that the second member of the SPCA family appears from Amphibia onward and is thus absent in fish.

Cell-Specific Expression. In human tissues, SPCA2 expression is more constrained than that of SPCA1, suggesting a more specialized physiological function of the former. Its mRNA is most abundant in the various segments of the gastrointestinal tract, in trachea, thyroid, salivary gland, mammary gland, and prostate.[176] Hence, SPCA2 appears most abundantly expressed in some cells endowed with an active secretion system like the mammary-gland cells during lactation[204] and the mucin-secreting goblet cells in human colon.[176,205] However, reported SPCA2 expression in keratinocytes, neutrophils, and hippocampal neurons does not fit this picture. These data on mRNA expression should, however, be confirmed at the protein level. So far, the presently available antibodies could only demonstrate SPCA2 expression in cultured hippocampal neurons,[177] in the colon,[176] in the secretory acini of the mouse mammary gland,[204] and in neutrophil granulocytes.[206]

Further arguments for the housekeeping role of SPCA1 and the specialized role of SPCA2 were supplied by Faddy et al.,[204] who showed a ubiquitous distribution of SPCA1 in all cells in mouse lactating mammary tissue whereas SPCA2 was only expressed in the milk-secreting luminal epithelial cells.

Subcellular Localization. The precise subcellular localization of SPCA2 is not completely unambiguous. In human goblet cells, both SPCA2 and SPCA1 colocalized with Golgi markers in a compact structure near the apical pole of the nucleus.[176] Also, upon heterologous expression in COS-1 cells, SPCA2 appeared in the Golgi area.[199] In cultured mouse hippocampal neurons, however, SPCA2 staining showed a punctate distribution in the cell body and in the dendrites.[177] Although in neurons the Golgi apparatus does in general appear as a more fragmented structure deviating from the ribbon-like juxtanuclear position observed in other cells, SPCA2 only partially colocalized with the *trans*-Golgi marker TGN38. It was therefore argued that in hippocampal neurons SPCA2 is at least partially localized in downstream, post-Golgi segments of the secretory pathway.[177]

4.4. Kinetic Properties of SPCAs

Detailed analyses on both the overall reaction cycle and the partial reaction steps of all SPCA1 isoforms and of SPCA2 in comparison with the reactions in SERCA1 are available.[193,207] SPCAs and SERCAs display distinct kinetic parameters, providing insight in the specific concentration dependences for the different substrates (Ca^{2+}, ATP, inorganic phosphate) and inhibitors (vanadate or thapsigargin), the different maximum turnover rates, and the different sensitivity to modulation by H^+ and K^+ ions of the E2-P dephosphorylation.

There are four human SPCA1 splice variants of which SPCA1c is a nonfunctional variant. The functionally active SPCA1a, SPCA1b, and SPCA1d displayed very high apparent affinities for Ca^{2+} ($K_{0.5}$ = 9−10 nM) relative to yeast SPCA ($K_{0.5}$ = 70 nM) or SERCA1a ($K_{0.5}$ = 284 nM). Also SPCA2 had a high apparent affinity for Ca^{2+} ($K_{0.5}$ = 25 nM). Depending on the specific isoform, the maximal turnover rates are 3.2- to 6.4-fold lower than that of SERCA1a (130 s^{-1}). SPCA2 has an almost 2-fold enhanced turnover rate relative to the human SPCA1 isoforms. In comparison to SERCA1a, SPCA1 isoforms are characterized by slower rates of phosphorylation.

The low turnover rate and high apparent affinity for Ca^{2+} of the SPCA1 pumps could in principle represent necessary adaptations to function in the Golgi compartments. A higher rate of ion accumulation is not required in the Golgi because it does not take part in rapid cytosolic Ca^{2+} signaling, and

Table 1. Overview of Different Inhibitors of Metazoan Intracellular Ca^{2+}-ATPases[a]

ATPase	assay	experimental system		potency	ref
		Thapsigargin			
SERCA1	$^{45}Ca^{2+}$ uptake	overexpression	COS	max inhibition = 25−100 nM	434
	% phosphorylation	overexpression	HEK	IC_{50} = 0.031 nM	193
	ATPase activity	overexpression	COS	K_i = 0.21 nM	147
SERCA2	$^{45}Ca^{2+}$ uptake	overexpression	COS	max inhibition = 25−100 nM	434
	ATPase activity	overexpression	COS	K_i = 1.3 nM	147
SERCA3	$^{45}Ca^{2+}$ uptake	overexpression	COS	max inhibition = 25−100 nM	434
	ATPase activity	overexpression	COS	K_i = 12 nM	147
SPCA1	% phosphorylation	overexpression	HEK	IC_{50} = 28 μM	207
SPCA2	% phosphorylation	overexpression	HEK	IC_{50} = 2 μM	207
		Cyclopiazonic acid			
SERCA1	ATPase activity	overexpression	COS	K_i = 90 nM	147
SERCA2	$^{45}Ca^{2+}$ uptake	A7r5,	16HBE14o-,COS	IC_{50} = 0.7−1.6 μM	211
	ATPase activity	overexpression	COS	K_i = 2.5 μM	147
SERCA3	ATPase activity	overexpression	COS	K_i = 0.6 μM	147
SPCA1	$^{45}Ca^{2+}$ uptake	A7r5,	16HBE14o-,COS	IC_{50} = 165−337 μM	211
SPCA2	N.D.	N.D.		N.D.	
		2,5-Di-*tert*-butyl hydroquinone			
SERCA1	ATPase activity	overexpression	COS	K_i = 7 μM	147
SERCA2	$^{45}Ca^{2+}$ uptake	A7r5,	16HBE14o-,COS	IC_{50} = 1−1.4 μM	211
	ATPase activity	overexpression	COS	K_i = 2.6 μM	147
SERCA3	ATPase activity	overexpression	COS	K_i = 1.7 μM	147
SPCA1	$^{45}Ca^{2+}$ uptake	A7r5,	16HBE14o-,COS	IC_{50} = >1 mM	211
SPCA2	N.D.	N.D.		N.D.	

[a] Different known inhibitors of metazoan intracellular Ca^{2+}-ATPases are summarized with their inhibitory concentration, the experimental system used, and the references (N.D. = not determined)

in most cells the depletion of Ca^{2+} occurs only via the slower process of forward trafficking of Ca^{2+}-rich vesicles down the secretory pathway. On the other hand, the higher turnover rate of SPCA2 in secretory cells might adapt these cells for more intense trafficking of Ca^{2+}-rich vesicles than in nonsecretory cells. The relatively high apparent affinity for Ca^{2+} displayed by SPCAs with $K_{0.5}$ values below the resting cytosolic [Ca^{2+}] ensures that the refilling process of the Golgi complex with cytosolic Ca^{2+} occurs continuously, even in the absence of transient rises in cytosolic [Ca^{2+}].

4.5. SPCA Inhibitors

Unfortunately, there are currently no known specific inhibitors of the SPCAs.[20] For functional studies, one is limited to assessing the active transport in conditions where all other P-type Ca^{2+}-transport ATPases are inhibited.[196,208] Therefore, it is imperative to know the sensitivity of SPCAs for the classical inhibitors of P-type Ca^{2+}-transport ATPases.

Already during the initial characterization of Pmr1, its sensitivity for thapsigargin was tested. Pmr1 was found to be insensitive to thapsigargin at concentrations of up to 5 μM,[209] whereas the inhibitory concentrations for SERCA are in the (sub)-nanomolar range. Since then, this insensitivity has been used extensively to discriminate between SERCA-dependent and SPCA-dependent Ca^{2+} stores.[210] Whereas SERCA1a shows a K_d value for thapsigargin of 0.03 nM, SPCA1d had a K_d of 28 μM (Table 1). Thapsigargin sensitivity of SPCA2, on the other hand, was 1 order of magnitude higher ($K_d = 2\ \mu$M). Nonetheless, SPCA2 is still considered to be thapsigargin-insensitive.[207]

Additionally, SPCA1 was also found to be 2 orders of magnitude less sensitive to cyclopiazonic acid and 2,5-di-*tert*-butyl hydroquinone[211] (Table 1). Like all P-type ATPases, SPCAs are inhibited by vanadate.[209] However, their affinity for vanadate is 2- to 5-fold lower when compared to SERCA1a.[193]

Another pharmacological tool that can be used to discriminate between SERCA-dependent and SPCA-dependent stores is bisphenol, a phenolic antioxidant. At low concentration (∼10 μM), it is believed to inhibit predominantly SERCA-dependent stores, while at higher concentration (∼40 μM), it inhibits both the thapsigargin-sensitive and thapsigargin-resistant components of Ca^{2+}-ATPase activity, showing that this compound inhibits both SERCA and SPCA pumps.[196,212]

4.6. Function of SPCAs

4.6.1. *pmr1* Mutants in Yeast

While homozygous null mutations in the *ATP2C1* gene encoding SPCA1 seem to be unviable in mammals,[213] they are tolerated in lower eukaryotes, including fungi and *C. elegans*,[214,215] where compensatory mechanisms presumably suffice to allow viability. A particularly tractable model for understanding such mechanisms is the yeast orthologue Pmr1. *pmr1* mutants in yeast display pleiotropic changes in Ca^{2+}-dependent growth,[197] secretion of unprocessed proteins,[197] outer-chain glycosylation,[214] tolerance to salt,[216] cell shape,[217] virulence[172] and viability.[218] The identification of diverse *pmr1* mutant phenotypes in yeast has been invaluable in guiding studies on the role of metazoan SPCA orthologues. Some of these studies will be reviewed below.

4.6.2. RNA Interference

Van Baelen et al. used RNA interference to further clarify the role of SPCA1 in HeLa cells.[219] Luminal [Ca^{2+}] measurements using Golgi-targeted aequorin showed that endogenous SPCA1 was responsible for Ca^{2+} uptake in a subcompartment of the Golgi. Upon knock-down, histamine still induced baseline Ca^{2+} oscillations, indicating that the SPCA1-containing Ca^{2+} store was not needed to set up oscillations. However, the frequency of the Ca^{2+} oscillations was reduced.

SPCA1 seems to be an important component of Ca^{2+} signaling in insulin-secreting cells.[220] Knock-down of SPCA1

partially diminished Ca^{2+} uptake into the ER and in dense-core secretory vesicles, increased Ca^{2+} influx through L-type Ca^{2+} channels, and increased the response to glucose. The shape, duration, and decay rate of Ca^{2+} oscillations in response to glucose plus tetraethylammonium were modified.

The same approach in cell lines expressing misfolded proteins revealed defects in protein processing and degradation specifically of glycosylated misfolded proteins. Furthermore, SPCA1 deficiency made cells hypersensitive to ER stress induced by tunicamycin.[221]

Knock-down of SPCA1 in *C. elegans* rendered the worms highly sensitive to Ca^{2+}-deficient and Mn^{2+}-enriched conditions and made them resistant to oxidative stress.[215] These defects are reminiscent of the mutant phenotype observed in yeast, as discussed earlier.

Using a genetically transmissible RNA-interference strategy in *Drosophila*, Southall et al. also showed aberrant Ca^{2+} signaling and abolished neuropeptide-stimulated diuresis in the Malpighian tubes of transgenic flies.[166]

Mutations in one copy of the *ATP2A2* gene encoding SERCA2 cause Darier disease in humans.[92] SPCA1 in the keratinocytes of these patients can compensate for the partial loss of SERCA2 function and is sufficient to blunt the response to raises in extracellular [Ca^{2+}]. Knock-down of SPCA1 in these Darier cells therefore resulted in a diminished viability, suggesting that compensatory up-regulation maintains viability.[222]

4.6.3. SPCA1 Mouse Models

The description of the phenotype of *SPCA1*$^{-/-}$ mice by Okunade and co-workers represented a hallmark for the SPCA field.[213] Homozygous mutant mice died in utero before gestation day 10.5. They exhibited growth retardation and had an open rostral neural tube. The Golgi membranes were dilated, were expanded in amount, and had fewer stacked leaflets as shown by electron microscopic analysis. Also the number of Golgi-associated vesicles was increased, although processing and trafficking of proteins in the secretory pathway was apparently normal. Apoptosis was increased and a large increase of cytoplasmic lipid was observed, consistent with impaired handling of lipid by the Golgi complex. The authors introduced the concept of Golgi stress to summarize these defects.[213]

Adult SPCA1 heterozygous mice had an increased incidence of squamous cell tumors of epithelial cells of the skin and esophagus.[213] Also SERCA2 heterozygous mice developed such tumors.[91] The development of squamous cell tumors in aged *ATP2A2*$^{+/-}$ and *ATP2C1*$^{+/-}$ mice indicates that SERCA2 and SPCA1 haploinsufficiency predisposes murine keratinocytes to neoplasia. The possible links between Ca^{2+}-transporting proteins and cancer have been reviewed in detail by Monteith et al.[223]

5. Other Ca^{2+}- and Mn^{2+}-Transporting P-type ATPases

Transition metals are important enzyme cofactors. High concentrations of redox-active metals like Cu, Zn, and Mn, however, can disturb normal cellular activity and, therefore, be toxic. Besides pumps like SERCAs or PMCAs that only transport Ca^{2+}, and pumps like SPCAs that transport Ca^{2+} or Mn^{2+} as their normal physiological metal ion, there exists a large subfamily of P_{1B} ATPases that transport heavy or transition metals (see refs 224 and 225 for excellent reviews). Also to this subfamily belong the mammalian Cu^{2+}-ATPases encoded by the *ATP7A* and *ATP7B* genes that, when mutated or inactivated, lead to, respectively, Menkes disease and Wilson disease.[226] Both enzymes transport Cu^{2+} from the cytosol into the secretory pathway, and hence, they serve a dual purpose, i.e., they prevent overload of the cytosol with Cu^{2+} and supply the secretory pathway with a cofactor that is necessary for many enzymes. Whereas Menkes disease is a fatal disorder due to disturbed function of many copper-dependent enzymes, Wilson disease represents a form of copper toxicosis with accumulation of toxic levels of the metal in liver and brain.[226] The P_{1B} pumps show eight transmembrane segments, unlike the P_2 pumps that comprise 10 transmembrane segments. They lack the transmembrane segments corresponding to TM7-10 of the P_2 pumps but have two additional transmembrane segments at their N-terminus. Characteristically, P_1 pumps also possess additional N- and C-terminal metal-binding domains. Because of the chemical similarities of the transition-metal ions, these pumps can often also transport nonphysiological substrates including Mn^{2+} or even Ca^{2+}.[225] Although this subfamily is deeply rooted in the prokaryotic world, many representatives are found in eukaryotes, particularly in plants, where the largest variety of transition-metal pumps is present.[224,225] These pumps transport Cu^{+}, Cu^{2+}, Ag^{+}, Zn^{2+}, Cd^{2+}, Co^{2+}, and Pb^{2+} and can be subdivided in 6 clusters: $P_{1B\text{-}1}$ to $P_{1B\text{-}6}$, which fall into two groups: pumps transporting predominantly Zn^{2+}, Cd^{2+}, Pb^{2+}, or Co^{2+} and pumps transporting Cu^{+} and/or Ag^{+}. In *A. thaliana*, AtHMA2−4 are thought to be Zn^{2+} transporters whereas AtHMA5−8 would act as Cu^{+}-ATPases.[225] AtHMA1 is the most divergent member of the plant P_{1B} ATPases and its normal substrate is uncertain. Besides transporting metals like Zn^{2+}, Cu^{2+}, Cd^{2+}, and Co^{2+}, AtHMA1 can also transport Ca^{2+} with a high apparent affinity (0.37 μM) and, most remarkably, it is strongly inhibited by thapsigargin (IC_{50} = 16.7 nM).[150]

6. Role of Ca^{2+} and Mn^{2+} in the ER and Golgi Apparatus

Both the SERCAs and the SPCAs actively transport Ca^{2+} into the lumen of the ER and the Golgi apparatus. Real-time [Ca^{2+}] measurements in the lumen of these compartments with targeted Ca^{2+}-sensing luminescent or fluorescent proteins or with low-affinity fluorescent Ca^{2+} probes are now available.[227] The free resting [Ca^{2+}] in the ER is in the range of a few hundred micromolar.[228,229] This high [Ca^{2+}] is preserved in the ER-Golgi intermediate compartment (ERGIC), in the various Golgi subcompartments and *trans*-Golgi network, and finally in the secretory vesicles. Direct [Mn^{2+}] measurements in the lumen of the Golgi apparatus are not yet possible. It is likely, however, that this compartment contains a high [Mn^{2+}] because its SPCA pumps also transport Mn^{2+}. The role of Ca^{2+} and Mn^{2+} in the lumen of these compartments will be reviewed.

6.1. Protein Glycosylation

N-linked glycosylation involves the addition of a core oligosaccharide unit of three glucoses, nine mannoses, and two *N*-acetylglucosamines ($Glc_3Man_9GlcNAc_2$) to the terminal amino group of asparagine. The core is assembled by glycosyltransferases on a membrane-bound dolichylpyrophosphate and brought by an oligosaccharyltransferase associated with the translocon complex to the growing

polypeptide chain in the ER.[230] The two outermost glucose residues are removed by glucosidase I and II, and the remaining $Glc_1Man_9GlcNAc_2$ oligosaccharide binds with its terminal glucose and three underlying mannose residues to the lectins calnexin, an integral membrane chaperone, and calreticulin, its soluble luminal counterpart. This binding requires a high [Ca^{2+}] and is significantly reduced when Ca^{2+} stores are empty.[231] Ca^{2+} also affects the structure of both lectins since its absence is associated with reduced melting temperature and with acquisition of protease sensitivity.[232] The acidic C-terminal region of calreticulin furthermore binds Ca^{2+} with high capacity and is involved in Ca^{2+} storage in the lumen of the ER.[233,234] Calnexin and calreticulin also form a Ca^{2+}-dependent complex with the ERp57 thiol disulfide oxidoreductase[235] and promote disulfide-bond formation of the glycoprotein. Folding and post-translational modification of nonglycosylated proteins involve other Ca^{2+}-binding chaperones including endoplasmin, the protein-disulfide-isomerase family of proteins (PDI, ERp72), and BiP.[231] Ca^{2+} also promotes folding by weakening electrostatic interactions and amplifying van der Waals interactions,[236] and by promoting subunit assembly and proteolytic clipping.[237] The glycoprotein is then released from calnexin/calreticulin, and subsequent cleavage of the innermost glucose by glucosidase II prevents further interaction with calnexin/calreticulin.[238] Incompletely folded glycoproteins are reglucosylated by a glucosyltransferase and enter another round of calnexin/calreticulin binding and disulfide-bond formation.[239] UDP-glucose (UDP, uridine diphosphate), the substrate for reglucosylation, enters the ER lumen via an UDP-glucose/UMP-exchanger. UMP (uridine monophosphate) is produced from UDP generated by the glucosyltransferase by either a soluble UDPase requiring Ca^{2+}, Mg^{2+}, or Mn^{2+}, or a membrane-associated UDPase requiring only Ca^{2+}. The remaining $Man_9GlcNAc_2$ chain in the completely folded glycoprotein is then trimmed to $Man_8GlcNAc_2$ by an ER-specific mannosidase with a coordinated Ca^{2+} that is essential for its activity.[240,241] The glycoprotein then goes to the Golgi compartment in a process assisted by mannose-binding lectins. Mannosidases in the Golgi apparatus further shorten the glycan chain to $Man_5GlcNAc_2$. One *N*-acetylglucosamine is then added, two additional mannoses are removed, and a new terminal sugar is added by various glycosyltransferases.

O-linked glycosylation of the mucin type, which is the most abundant form in higher eukaryotes, is characterized by the binding of *N*-acetylgalactosamine to the hydroxyl group of a serine/threonine residue on a target protein. In contrast to *N*-linked glycosylation, *O*-linked glycosylation does not begin with the transfer of an oligosaccharide from a dolichol precursor, but with the addition of a single *N*-acetylgalactosamine by polypeptide *N*-acetylgalactosaminyltransferases.[242,243] The type of enzyme determines whether *O*-linked glycosylation is initiated in subregions of the ER, in the ERGIC, or in the various Golgi subcompartments. Subsequent elongation of this structure by downstream glycosyltransferases yields an array of eight distinct "core" glycans that can be further modified by many of the glycosyltransferases resident in the Golgi. Some specific types of *O*-glycosylation deviate from the common mucin-type pathway, e.g., the *O*-linked binding of fucose and glucose to epidermal growth factor homology regions in several proteins,[244] and of galactose to hydroxylysine in collagen.[245] Another type of *O*-linked glycosylation is the addition of *N*-acetylglucosamine to serines and threonines of cytoplasmic and nuclear proteins, which represents a reversible regulatory modification.[246,247]

Glycosyltransferases transfer a monosaccharide from an activated sugar donor to the hydroxyl group of another sugar, an amino acid, or a lipid.[248] Glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, arabinose, glucuronic acid, galacturonic acid, and xylose are activated as UDP-derivatives; mannose and fucose are activated as GDP-derivatives; and sialic acid is activated as a CMP-derivative (GDP, guanosine diphosphate); (CMP, cytidine monophosphate).[249] The length of their hydrophobic membrane-spanning region fits with the thickness of the Golgi membrane and, therefore, keeps most of these enzymes anchored in the Golgi compartment, but also other mechanisms are involved.[250] Many glycosyltransferases require Mn^{2+} for activity.[251] Examples include galactosyltransferases,[252,253] *N*-acetylglucosaminyltransferases,[254] mannosyltransferases,[255] glucuronyltransferases,[256] arabinofuranosyltransferases,[257] some fucosyltransferase-family members,[258,259] and *N*-acetylgalactosaminyltransferases.[260] In glycosyltransferases of the A superfamily,[261] which includes most of the enzymes found in the ER and Golgi apparatus, Mn^{2+} interacts with one or both acidic residues of a three-residue D-x-D, E-x-D, or equivalent motif.[249,253,262,263] Glycosyltransferases of the B superfamily do not have this motif and do not need Mn^{2+} for activity.[249,262,264] However, some enzymes of the A superfamily lack the D-x-D or E-x-D motif and do not require Mn^{2+}, while some enzymes of the B superfamily do require Mn^{2+}.[249] Structural studies on several of these enzymes revealed that the sequential binding of Mn^{2+}, if required for catalysis, and the sugar-nucleotide results in a conformational change that creates the acceptor-binding site. After sugar transfer, the product is ejected from the binding site, after which the enzyme reverts to the open conformation, facilitating release of the UDP−metal complex.[249]

We mentioned earlier that SPCA-type pumps play an important role in the accumulation of Ca^{2+} and Mn^{2+} into the Golgi apparatus. Down-regulation of SPCAs therefore interferes with glycosylation. FRT-thyroglobulin cells treated with SPCA1 siRNA show defects in *N*-linked glycosylation of newly synthesized thyroglobulin.[221] *pmr1* mutants of *S. cerevisiae* secrete defective *N*-linked glycosylated invertase,[197,214] deficient *O*-glycosylated chitinase,[265] and a nonglycosylated variant of human plasminogen activator,[160] and are defective in carbohydrate trimming of $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$.[266] Disruption of Pmr1 in *C. albicans* also results in *N*- and *O*-linked-glycosylation defects.[172] Pmr1 disruption in *P. pastoris* results in the secretion of a human serum albumin/interferon-α2b fusion protein with incomplete disulfide-bridge pairing.[267]

6.2. ER-Associated Degradation

Polypeptides that fail to acquire their native structure are eventually removed by ER-associated degradation (ERAD). They return to the cytosol in a process possibly mediated by EDEM (ER-degradation enhancing α-mannosidase-like protein) and Derlin, for ubiquitination and degradation by the proteasome.

Luminal Ca^{2+} and/or Mn^{2+} are involved in ERAD. Cells that overexpress the misfolded "cog" mutant of thyroglobulin show a major defect in ERAD of this protein when treated with siRNA against SPCA1.[221] *pmr1* mutants of *S. cerevisiae* are defective in the degradation of the misfolded carboxypeptidase-Y glycoprotein.[265,266] The ERAD defect after

Pmr1 knock-down only applies to glycosylated proteins and not to nonglycosylated proteins and is correlated with defective trimming of the $Man_9GlcNAc_2$ chain to $Man_8GlcNAc_2$[266] There are various explanations why Pmr1 in the Golgi complex may affect the ER-localized ERAD. *First*, Ca^{2+} can be transported from the Golgi apparatus to the ER via COPI-coated vesicles involved in Golgi-ER retrograde transport.[268] However, ER Ca^{2+} uptake proceeds unperturbed after a block in Golgi-to-ER retrograde transport.[269] *Second*, since Pmr1 is first synthesized in the ER, it could exert its function before being transported to the Golgi apparatus.[265] ER [Ca^{2+}] measurements revealed that a fraction of Pmr1 in *S. cerevisiae* is indeed retained in the ER,[269] and subcellular fractionation experiments in *S. cerevisiae* consistently found a small fraction of Pmr1 associated with ER markers.[197,209] Furthermore, GFP-tagged Pmr1 in *S. pombe* appears to be localized predominantly in the ER rather than in the Golgi apparatus.[217] Since hemagglutinin-tagged Pmr1 was localized in the Golgi and GFP-tagged Pmr1 was localized in the ER under certain experimental conditions, it is possible that Pmr1 may be mislocalized or may accumulate in the ER owing to GFP tagging. *Third*, a retrotransport of the Golgi misfolded proteins themselves back to the ERAD machinery of the ER may also explain why Pmr1 in the Golgi complex affects the ER-localized ERAD.

6.3. Sulfatation

Golgi-resident sulfotransferases transfer a sulfate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to a tyrosine residue in a protein or to an alcohol or amino group in glycoconjugates.[270] Mn^{2+} enhances the activity of tyrosylprotein sulfotransferases[271,272] and carbohydrate sulfotransferases.[273−275] Some sulfotransferases acting on mucins have a tissue distribution restricted to the lung[276] or the small intestine and colon,[277] i.e., tissues rich in mucin-secreting cells. Interestingly, this distribution correlates with the expression pattern of SPCA2 and with the relatively higher expression levels of SPCA1.

6.4. Proteolytic Processing

The biosynthesis of peptide hormones often begins with mRNA translation into a large, inactive precursor polypeptide. Proteolytic processing then involves the action of proprotein/prohormone convertases in the *trans*-Golgi network and in the regulated and constitutive secretory pathways.[278] This family of serine endoproteases includes furin, prohormone convertase 1, prohormone convertase 2, and many others.

The proprotein/prohormone convertases are synthesized as inactive zymogens because their catalytic domain is blocked by their N-terminus. Furin activation requires a first cleavage of this N-terminal inhibitory peptide in the ER and a second cleavage in the *trans*-Golgi network requiring millimolar [Ca^{2+}] and an acidic pH.[279] Propeptide cleavage in the prohormone convertase 1, in contrast, requires only micromolar [Ca^{2+}].[279] Once activated, the proprotein/prohormone convertases cycle between the *trans*-Golgi network, the cell surface, and the endosomal compartments.

The reported crystal structures of several proprotein/prohormone convertases revealed two Ca^{2+}-binding domains. One site is important for the structural stability of the protease; the other one is important for the structure of the active site.[280−282] The various convertases seem to differ in the Ca^{2+} requirement for optimal activity.[283−286]

pmr1 mutants of *S. cerevisiae* show an impaired proteolytic processing of pro-alpha factor. This phenotype may reflect a reduction in the activity of Kex2, a Ca^{2+}-dependent Golgi-localized endoprotease.[197]

6.5. Trafficking

Ca^{2+} is needed for the transport among the various compartments. Some of these effects occur from the lumen of these compartments. Cells of *SPCA1*$^{-/-}$ mouse embryos have more dense vesicles on the *cis*-side of the Golgi, suggesting that trafficking of vesicles between the ER and the Golgi apparatus is affected.[213] Inhibition of SERCA-dependent Ca^{2+} uptake with thapsigargin blocks both the anterograde[287] and retrograde transport between the ER and the Golgi apparatus.[288] ERGIC-53 in the ERGIC compartment, which recycles between the Golgi apparatus and the ER, is a Ca^{2+}-dependent lectin.[289] The cation-dependent mannose-6-phosphate receptor, a component of the lysosomal targeting system, contains a carbohydrate-binding site in which Mn^{2+} interacts with the phosphate group of mannose-6-phosphate.[290]

Stored Ca^{2+} may also affect transport after being first released. Anterograde and retrograde transport of cargo between the Golgi stacks was more efficiently blocked by the fast Ca^{2+} buffer BAPTA than by the slow Ca^{2+} buffer EGTA, suggesting that Ca^{2+} may be delivered locally, perhaps via constant leakage from the Golgi complex itself.[70,291−293] Ca^{2+} in secretory vesicles may be the source of Ca^{2+} for creating a specific Ca^{2+} microdomain that controls granule motion and exocytosis.[294,295]

Disruption of Pmr1 in yeast often results in enhanced protein secretion. Null mutations in the *PMR1* gene in *S. cerevisiae* disrupted the sorting and retention of heterologous secretory proteins normally destined for degradation in the lysosome, resulting in an enhanced secretion of, e.g., prochymosin, a bovine growth hormone, and a nonglycosylated variant of human urinary plasminogen activator.[160,197,214] Disruption of Pmr1 in *S. cerevisiae* and *H. polymorpha* disrupted the sorting of carboxypeptidase Y from the late Golgi compartments to the vacuole, resulting in an enhanced secretion of this protein in the culture medium.[218,265] Disruption of Pmr1 in *H. polymorpha* decreased intracellular aggregation of human urokinase and enhanced its secretion.[218] Disruption of Pmr1 in *K. lactis* led to enhanced secretion of acid phosphatase, recombinant human serum albumin, and α-glucoamylase as a result of increased translation and/or secretion efficiency.[296] For biotechnologists, *PMR1*-gene disruption, e.g., in *P. pastoris*, can become an effective strategy to increase the secretion capacity of heterologous proteins during large-scale fermentation for commercial protein production.[297] Mutants of ECA3, a Golgi-localized Ca^{2+},Mn^{2+}-ATPase in *A. thaliana*, secreted more total protein and exhibited more peroxidase activity than wild-type plants, indicating that perturbation of Ca^{2+} and/or Mn^{2+} homeostasis in the stores perturbs protein secretion.[14,298]

The effect of disruption of the yeast Pmr1 on protein secretion depends on the marker protein analyzed. Disruption of the *PMR1* gene in *S. cerevisiae* resulted in the expected increased secretion of bovine prochymosin, while that of the plant protein thaumatin could not be improved to any significant extent.[299] Such various responses were also observed in *Y. lipolytica*.[300] The secretion of 32 kDa mature

alkaline extracellular protease decreased, while incompletely processed 52 and 36 kDa precursors were secreted. Also, very low levels of rice α-amylase secretion were observed, probably because the decreased luminal [Ca^{2+}] interfered with the stabilization of the tertiary structure of the enzyme. The secretion of *Trichoderma reesei* endoglucanase I was not influenced.

6.6. Milk Production

Synthesis of lactose, the sugar in milk, involves the interaction of β-1,4-galactosyltransferase 1 with α-lactalbumin to form the lactose-synthase complex that transfers galactose from UDP-galactose to glucose.[301] β-1,4-galactosyltransferase 1 is a *trans*-Golgi enzyme that becomes up-regulated during lactation.[302] α-Lactalbumin is only expressed in the mammary gland and more specifically during lactation.[303] Lactose synthesis follows a sequentially ordered mechanism in which Mn^{2+} binding to β-1,4-galactosyltransferase is followed by UDP-galactose binding, which creates a stable complex to which α-lactalbumin and glucose bind. After catalysis, α-lactalbumin and lactose are released, followed by a conformational change and the release of Mn^{2+} and UDP.[301] Lactose accumulates within the lumen of the Golgi membranes and comes into the milk by exocytosis from secretory vesicles derived from the Golgi apparatus. The galactosyltransferase is also secreted during this process.

Casein is an important milk protein. Its phosphorylation allows Ca^{2+} binding and cross-linking of casein polypeptides by calcium phosphate, which is essential for the formation and stability of the typical casein-micelle structure.[304] Casein is phosphorylated in the lumen of the Golgi apparatus. The responsible casein kinase specifically recognizes the consensus sequence S-x-E/pS, which differs from the consensus sequences of either casein kinase 1 (pS-x-x-S/T) or casein kinase 2 (S/T-x-x-E/D/pS), which are also designated casein kinases because their enzymatic activity has been initially detected using casein as a substrate. The molecular identity of the Golgi casein kinase has not yet been fully elucidated.[305,306] The Golgi casein kinase is expressed not only in the mammary gland but also in the liver and endocrine cells[307,308] and represents a pleiotropic kinase phosphorylating a wide diversity of secreted proteins. Casein phosphorylation mediated by casein kinase in vitro[309,310] is stimulated by Ca^{2+} and more effectively by Mn^{2+}. Ca^{2+} depletion of intact acini with a Ca^{2+} ionophore had no effect on an early stage of casein phosphorylation but partially inhibited a later phase.[311] Ionomycin however works suboptimally in acidic compartments, so the possibility remains that, in these experiments, the downstream Golgi compartments contained some residual Ca^{2+}.

Ca^{2+} in milk is needed for the rapid calcification of bones and teeth of the neonate.[312] The total milk [Ca^{2+}] exceeds 60 mM in rapidly growing species like mice and rats. Human breast milk[313] contains around 12 mM Ca^{2+}. It had been traditionally thought that all Ca^{2+} entered the milk through the Golgi pathway, where it was packaged with caseins, phosphate, citrate, and other Ca^{2+}-binding molecules and released into the milk by exocytosis of secretory vesicles.[312,314] We now know that PMCA expression increases dramatically during lactation in rat mammary tissue,[315] that mammary PMCA is primarily PMCA2b, and that PMCA2-null mice produce milk containing 60% less Ca^{2+} than wild-type mice.[316] PMCA2 is expressed in the apical membrane of secretory mammary epithelial cells during lactation and may play a role in enriching milk with Ca^{2+}. The ER lumen could offer a route for long-range Ca^{2+} propagation across the epithelial cell. Ca^{2+} travels through the ER lumen much more easily than through the cytosol.[317−319] The relative ease with which Ca^{2+} moves in the ER lumen and in the cytosol depends on the nature of the Ca^{2+} buffers in these two compartments, their relative mobilities, and the [Ca^{2+}].[320,321] Ca^{2+} in the cytosol binds to numerous high-affinity Ca^{2+}-binding proteins (e.g., calmodulin) and other molecules (e.g., ATP). The much lower affinity of intra-ER Ca^{2+} buffers in conjunction with the much higher luminal [Ca^{2+}] results in substantially higher Ca^{2+}-diffusion rates within the ER. Also, Ca^{2+} uptake by organelles (e.g., mitochondria) can contribute to the slower rate of diffusion in the cytosol. Intraluminal Ca^{2+} tunneling may occur in the lactating mammary gland as well. It is in this respect important that SERCA becomes up-regulated during pregnancy in the rat and mouse mammary gland.[322,323] If organellar Ca^{2+}-release channels would be preferentially localized in the apical zone, then Ca^{2+} would be released near the luminal surface of the cell in close proximity to the PMCA2 Ca^{2+} pumps. Energy-dispersive analytical X-ray scanning electron microscopy indeed revealed that the apical region of lactating rat mammary epithelial cells contains 4- to 5-fold more Ca^{2+} than other parts of the cell.[324] This area includes the Golgi apparatus and secretory vacuoles containing dense granules or casein micelles binding Ca^{2+}.

Both SPCA1 and SPCA2 are expressed in the Golgi apparatus of the lactating mammary gland.[205,315,325] SPCA2 levels increase over 15- to 35-fold during lactation, while SPCA1 increases only a modest 2- to 5-fold.[204,323] The up-regulation of SPCA2 during midpregnancy is likely to be hormonally regulated because SPCA2 transcription is increased by prolactin in human MCF-7 breast-cancer cells.[326] SPCA2 is only expressed in acinar cells, while SPCA1 as a housekeeping enzyme is also present outside the acini.[204] Excessive prepartum up-regulation of SPCA1 in cows may cause milk fever,[327] a metabolic condition characterized by a low plasma [Ca^{2+}]. SPCA1, SPCA2, and also PMCA2 expressions decline by 80−95% within 24 h of the start of mammary involution.[328]

6.7. TOR Signaling

TOR (target of rapamycin) is a highly conserved protein kinase that controls cell growth in response to nutrients. TOR in *S. cerevisiae* is found in two structurally and functionally distinct multiprotein complexes, TOR complexes 1 and 2. TOR proteins require Mn^{2+} as a cofactor for maximal activity.[329] Pmr1-dependent Mn^{2+} uptake into the Golgi apparatus inhibits TOR1 signaling.[330,331] Mn^{2+} indeed acted from the lumen of this compartment since the phenotype induced by loss of Pmr1 could be rescued by overexpression of Ccc1, a Mn^{2+} transporter localized to the Golgi and the vacuole. It is unclear, however, how luminal Mn^{2+} exerts its inhibitory action, since TOR1 is not found inside the Golgi.[332] It has been suggested that luminal Mn^{2+} may affect downstream effectors of TOR signaling, e.g., the sorting of nutrient permeases to the plasma membrane via Mn^{2+}-dependent mannosylation of sphingolipids.[330]

6.8. Regulation of Ca^{2+}-Transport Proteins

The sensitivity of Ca^{2+} release from muscle SR[333,334] and from neuronal ER is increased by its luminal Ca^{2+} load,

suggesting that modulation of Ca^{2+} signaling by luminal [Ca^{2+}] is a general phenomenon.[227] Both types of Ca^{2+}-release channels, the RyR and the *myo*-inositol-1,4,5-trisphosphate receptor (IP_3R), are regulated by the luminal [Ca^{2+}]. The open probability of RyRs incorporated into lipid bilayers increases if Ca^{2+} on the luminal side of the channel is elevated.[335−338] This stimulation could involve a Ca^{2+}-binding site on the luminal side of the channel[339,340] or occur via the Ca^{2+}-binding protein calsequestrin in complex with triadin 1 and junctin, since calsequestrin inhibits the RyR at low but not high luminal [Ca^{2+}].[341,342] Effects of luminal Ca^{2+} may also be due to Ca^{2+} passing through the channel and acting at the stimulatory cytoplasmic Ca^{2+} site.[343−345] Luminal Ca^{2+}-dependent changes in RyR2 gating are involved in Ca^{2+}-release termination and release refractoriness, processes essential for, e.g., normal rhythmic activity of the heart.[346]

The sensitivity of IP_3Rs to IP_3 is increased by a high luminal [Ca^{2+}] in some studies,[347−351] but not in others.[352−354] It is unclear whether this control operates over the physiological range of luminal [Ca^{2+}], since stores have to be extensively depleted before IP_3R sensitivity changes.[355−358] In contrast, single-channel IP_3R activity in planar lipid bilayers increased instead of decreased when [Ca^{2+}] at the luminal side of the channel declined,[359] but the divalent-cation-current carriers could have interacted with potential Ca^{2+}-binding sites on the luminal face of the receptor.[360] The effect of luminal [Ca^{2+}] on IP_3R activity might, just like for the RyR, arise from an interaction of released Ca^{2+} with cytosolic Ca^{2+}-binding sites.[361−363] Ca^{2+} may also act, however, from the lumen via the luminal Ca^{2+}-binding site[364] or via associated proteins. ERp44 protein-disulfide isomerase interacts with IP_3R1 in the ER lumen and inhibits channel activity.[365] The interaction between ERp44 and IP_3R1 becomes stronger at lower levels of store loading, and this could explain the inhibition of IP_3-induced Ca^{2+} release under these conditions.

Influx of extracellular Ca^{2+} into cells can be induced by depleting intracellular Ca^{2+} stores, a process referred to as capacitative or store-operated Ca^{2+} entry.[366] The STIM (stromal interaction molecule) proteins are believed to sense the Ca^{2+} content of the intracellular stores.[367,368] Upon store depletion, STIM1 redistributes within the ER approaching the plasma membrane and either directly or indirectly signals to plasma-membrane Orai proteins (or CRACMs), which represent an entirely new family of highly Ca^{2+}-selective Ca^{2+}-influx channels.[369,370] This process has been reviewed extensively[371,372] and will not be further considered here.

7. Mn^{2+} Toxicity

Mn^{2+} is abundantly present in the environment, but organisms appear to actively limit the concentration of this element in their bodies. However, Mn^{2+} is essential for a variety of cellular functions in mammals, some of which were described above (section 6). The small amounts of Mn^{2+} we need can easily be obtained via our diet, mainly from fruits and vegetables. Mn^{2+} deficiency, therefore, is rare in humans and only occurs in self-selected diets. Mn^{2+} deficiency causes alterations in bone metabolism[373] and susceptibility to epilepsy.[374] Conversely, excess of Mn^{2+} causes a Parkinson's disease-like syndrome known as Manganism.[375] Mn^{2+} can enter the body via two possible routes: (1) via the diet or (2) absorption through the respiratory and olfactory mucosa. The amount of Mn^{2+} in the diet is several orders of magnitude higher than the physiological need. However, since the amount absorbed through the gut is much lower, intoxication via the food is seldomly observed. Instead, in the case of Mn^{2+} intoxication, absorption through the respiratory system appears to be the main entrance pathway into the body. Often this occurs via occupational overexposure and chronic inhalation of airborne Mn^{2+} affecting miners, steel workers, welders, and workers in alkaline-battery production.[376] Also, environmental exposure to pesticides[377] containing Mn^{2+} or to combustion of fuel with methylcyclopentadienyl manganese tricarbonyl (MMT) additives[377,378] increases the population at risk for intoxication.

The liver is the main organ involved in the detoxification of Mn^{2+} via biliary excretion. Hence, liver damage as occurs in cirrhosis (by alcohol abuse or primary biliary cirrhosis) reduces Mn^{2+} elimination. Thus, there is often a correlation between liver diseases, Mn^{2+} accumulation in the brain, and encephalopathy.[379] Similarly, long-term parenteral nutrition can also result in Mn^{2+} toxicity, also causing neurological disorders, since Mn^{2+} bypasses the gut and liver control mechanisms via this route.[380]

The selective retention and slower elimination of Mn^{2+} in the brain in comparison with other tissues make the central nervous system the major target of Mn^{2+} poisoning. We will focus, therefore, on Mn^{2+} neurotoxicity.

7.1. Mn^{2+} Toxicity in the Brain

In contrast to Ca^{2+}, Mn^{2+} homeostasis in the brain is poorly understood. Most of the Mn^{2+} in the blood is bound to albumin, and only a small fraction exists as the free, hydrated ion.[381] Blood Mn^{2+} can be oxidized to Mn^{3+}, which is more toxic and reactive and immediately binds to transferrin.[382] Both Mn^{2+} and Mn^{3+} forms can cross the blood−brain and blood−cerebrospinal fluid barriers via transepithelial transport. Different mechanisms have been suggested to mediate the Mn^{2+} uptake into the barrier cells: Ca^{2+} channels,[383] Na^+/Ca^{2+}-exchangers,[384] or the divalent-metal transporter 1 (DMT1).[385] Mn^{3+} bound to transferrin is taken up by transferrin receptor-mediated endocytosis.[386] DMT1 is highly expressed in brain,[387] and an increase in expression has been reported after exposure to excess Mn^{2+}. However, none of these transporters are specific Mn^{2+} transporters. Even less is known regarding the mechanism by which Mn^{2+} again leaves the cells of the capillary endothelium or the choroid endothelium, nor regarding the mechanisms of Mn^{2+} elimination from neurons or glial cells. With respect to this, it is clear that SPCA can switch from transport of Ca^{2+} to transport of Mn^{2+}. In vitro assays of SPCA-dependent Ca^{2+} transport in systems overexpressing SPCA[177,198] and in neural membrane vesicles derived from pig and mouse[208,388] show a competition between Ca^{2+} and Mn^{2+} for transport. Therefore, it can be hypothesized that, like in *S. cerevisiae*, in neurons SPCA can pump excess cytosolic Mn^{2+} into the Golgi complex for its removal via the secretory pathway.[186,389] SPCA is the only known ATPase in animals that can transport Mn^{2+} with high affinity.[165,198] In rat brain it is prominently up-regulated following Mn^{2+} exposure.[390] In plants, the ECA3 Ca^{2+},Mn^{2+}-ATPase also plays a role in Mn^{2+} detoxification,[298] since *eca3* mutants are sensitive to high Mn^{2+}. It should be noted that removal of excess copper from the cell also occurs via the secretory pathway by a copper-transporting P-type ATPase located in the *trans*-Golgi network of hepatocytes. As discussed already in section 5, malfunction of this pump results in a copper

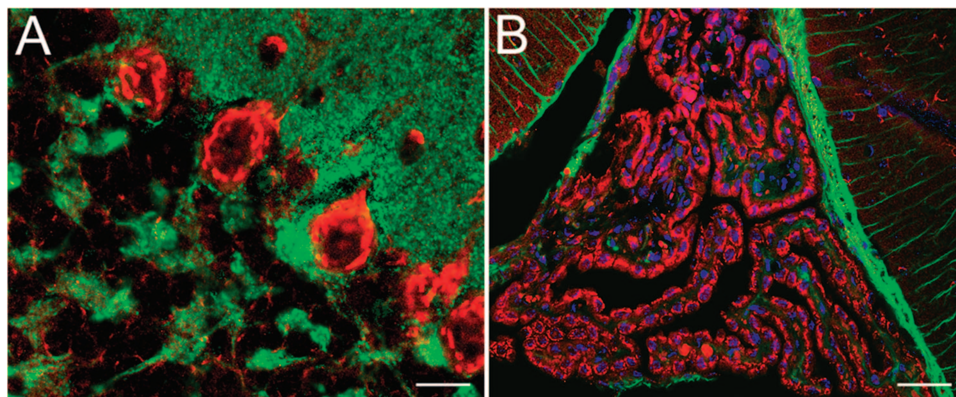

Figure 6. Distribution of SPCA1 in the adult mouse cerebellum. (A) Localization of SPCA1 (red) and synaptophysin (green) in the cerebellar cortex. (B) Distribution of SPCA1 (red) and the glial marker GFAP (green) in the choroid plexus of the fourth ventricle. Nuclei are visualized with Dapi staining (blue). Scale bars: (A) 15 μm; (B) 35 μm. More details can be found in manuscript by Sepulveda et al. in ref 388.

toxicosis known as Wilson disease.[391] The *ATP2C2* gene encoding SPCA2 has been excluded as the affected gene in a rare extrapyramidal motor disorder with accompanying hypermanganesaemia,[392] but unfortunately the authors did not include in their analysis the *ATP2C1* gene encoding the far more abundantly expressed neural SPCA1 isoform.

Manganism patients suffer from nervous-system dysfunction with alteration in motor coordination, memory loss, and psychological changes, as well as from fertility problems.[375] In the human brain, Mn^{2+} accumulates mainly in the basal ganglia.[393] Mn^{2+} accumulation can be traced by magnetic resonance imaging since Mn^{2+} is a potent T_1-shortening agent that causes contrast enhancement in T_1-weighted MRI images.[394] Thus, there is a correlation between the MRI-associated T_1-relaxation time and Mn^{2+} depositions.

The link between Mn^{2+} accumulation and neurodegeneration is still unclear. It has been reported that high levels of Mn^{2+} can inhibit tyrosine hydroxylation affecting dopamine synthesis,[375] interfere with Mg^{2+}-binding sites in many proteins,[395,396] alter iron homeostasis associated with neural oxidative damage,[397,398] and affect synaptic transmission by enhancing the release of neurotransmitters.[383,399] Mn^{2+} is also a cofactor of a number of key enzymes in the brain such as superoxide dismutase or glutamine synthetase, which are important, respectively, for countering oxidative stress and glutamate excitotoxicity,[400,401] i.e., additional causative factors of neural dysfunction. Therefore, increased cytoplasmic [Mn^{2+}] in astrocytes can in turn disrupt the activity of Mn^{2+}-sensitive enzymes such as glutamine synthetase.[402] Manganism symptoms can persist even several years after cessation of Mn^{2+} exposure.[403] Chelation therapy with ethylenediaminetetraacetic acid (EDTA), which forms stable chelates with metal ions that are subsequently excreted via the urine, and supplementation with levodopa (a drug for Parkinson's disease) are the current treatments.[392,404] However, these strategies show only limited benefits.

Mn^{2+} metabolism is also altered in other neuropathologies. Elevated blood [Mn^{2+}] was reported as a potential diagnostic marker for prion infection even before the onset of symptoms in scrapie and bovine spongiform encephalopathy.[405] In fact, Mn^{2+} binding to prion protein can potentiate the rate of prion-protein conversion to the abnormal isoform.[406]

Taking in account that neural cells require Mn^{2+} and Ca^{2+} ions for many different neural activities, including neural development, neural plasticity, and synaptic transmission, and that these ions also seem involved in aging, neurodegeneration, or apoptosis,[407,408] it is very important to understand the regulation of the cytoplasmic and luminal [Ca^{2+}] and [Mn^{2+}] in nerve cells. We will focus, therefore, on the role of ion pumps in these processes.

7.2. SERCAs and SPCAs in the Brain

7.2.1. Localization

SERCA2b represents the main SERCA isoform in the principal large neural cells (e.g., pyramidal cells of cerebral cortex and hippocampus, and cerebellar Purkinje cells) in different species. SERCA2b is localized in the ER of the soma and the dendritic arborization, ranging from primary dendrites to spiny branchlets.[409−412] Although other isoforms like SERCA2a[410,413−415] and SERCA3[416] have been found in Purkinje-cell bodies in a few species, they are weakly expressed in the other brain regions, if present at all. With respect to the SPCAs, the housekeeping SPCA1 constitutes the main isoform in nervous tissue. Neurons typically contain a more fragmented Golgi apparatus with several stacks distributed over the cell body that all contain SPCA1[208,388,417] (Figure 6A). The expression of SPCA2 in brain is less well-characterized, but its expression seems to be lower and less widespread than that of SPCA1.[176,177]

Although SPCA1 expression has been reported in different glial cultures,[417] present efforts to demonstrate SPCA- or SERCA-pump expression in glial cells in nervous tissue remained unsuccessful.[208,388,410] This is surprising since glial cells can be expected to tightly control Mn^{2+} homeostasis. Glial cells express the Mn^{2+}-requiring enzyme glutamine synthetase. It has been reported that glutamine synthetase-bound Mn^{2+} accounts for ~80% of the total Mn^{2+} content in the brain.[400] Glutamine synthetase is involved in the detoxification of brain ammonia and in the metabolic removal of the neurotransmitter glutamate.

SERCA2b and SPCA1 pumps have also been found in the epithelial cells of the choroid plexus (Figure 6B).[388,412] These cells are involved in the production and secretion of the cerebrospinal fluid that mechanically protects the brain and in which the [Ca^{2+}] is strictly regulated in ref 388

7.2.2. Function

Both SERCAs and SPCAs contribute to the Ca^{2+}-ATPase activity of microsomal membranes isolated from nervous tissue of different species.[196,208,388] SERCA activity appears to be involved in the regulation of the cytosolic [Ca^{2+}] in postsynaptic areas such as dendritic arborization.[419] SPCA

activity could be more focused on Ca^{2+}- and Mn^{2+}-dependent activities involved in the processing and trafficking of new proteins and membranes in the Golgi apparatus,[420] with special relevance during neural development. Brain development, including migration and functional maturation of neural precursors, as well as growth in size and complexity, have all been linked to the ontogeny of Ca^{2+} homeostasis.[421] Consequently, SERCAs and SPCAs are functionally upregulated during development[388,412] and are related to the maturation of specific neuronal types and the establishment of synaptic connections. The embryonic lethality of *SPCA1*$^{-/-}$ mice with abnormalities in the neural tube, growth retardation and Golgi stress,[213] suggests a specific role of SPCA1 in neural development. In fact, Mn^{2+} is an essential ion for normal prenatal and neonatal development of brain function as well as for the skeleton and the inner ear.[422]

Just like alterations in Mn^{2+} metabolism cause drastic effects in the nervous system (described above), also Ca^{2+} homeostasis is affected in many neurological disorders. Several ER- or Golgi-resident proteins subjected to Ca^{2+}-dependent processing such as presenilins,[423,424] or their corresponding processing enzymes like proprotein/prohormone convertases[424] or secretases,[425] are critically involved in neuropathologies like Alzheimer's disease. A variety of mechanisms including Ca^{2+} dysregulation cause fragmentation of the Golgi complex as one of the earliest and probably irreversible steps of neurodegeneration in amyotrophic lateral sclerosis, Alzheimer's disease, Creutzfeldt−Jacob disease, and spinocerebellar ataxia.[426] Also, Darier-disease patients lacking a functional copy of the SERCA2 gene are prone to develop neuropsychiatric disorders.[427] There are many excellent reviews on all these topics.[428,429] Thus, Ca^{2+} pumps should be considered as interventional targets for these neuropathologies with Ca^{2+} dysregulation.

Box 1. Genetic Diseases Involving SERCAs and SPCA

Brody myopathy (OMIM 601003) can be an autosomal-recessive disorder caused by mutations in the *ATP2A1* gene encoding SERCA1 in fast-twitch skeletal muscle. Patients suffer from painless muscle cramping and exercise-induced impairment of muscle relaxation.[430] The mutations may affect the splice donor site of intron 3 or give rise to premature stop codons resulting in a truncated protein.[61,431,432] Mutations in the *ATP2A1* gene similarly cause congenital pseudomyotonia in Chianina cattle,[62] with a lifelong history of exercise-induced muscle contracture, preventing animals from performing more intense muscular activities than a simple walk at a slow pace. Mutations in the *ATP2A1* gene also lead to congenital muscular dystonia 1 in Belgian Blue cattle.[63] Affected calves show impaired swallowing, fatigue upon stimulation or exercise, and muscle myotonia resulting in injurious falling and an inability to flex limbs. They usually die within a few weeks.

Darier disease (OMIM 124200) is an autosomal-dominant skin disease caused by missense mutations affecting one copy of the *ATP2A2* gene encoding SERCA2.[92] It is characterized clinically by warty papules and plaques in seborrheic areas (central trunk, flexures, scalp, and forehead), palmoplantar pits, and distinctive nail abnormalities. Histological examination shows a focal loss of adhesion between epidermal cells (acantholeysis) and an abnormal keratinization.

Hailey−Hailey disease (OMIM 169600) is an autosomal-dominant skin disease caused by the loss of one functional copy of the *ATP2C1* gene encoding SPCA1.[162,163] It is characterized by recurrent blisters and erosions in the flexural areas.[433] Histological examination shows numerous acantholeytic cells and a suprabasal type of blister formation.

8. Conclusions

We have reviewed the phylogeny, mechanism, regulation, and properties of the SERCA and SPCA pumps. We begin to understand their role in the Ca^{2+} and Mn^{2+} homeostasis in the ER, Golgi apparatus, and downstream secretory pathway. This role seems to be crucial for normal cell physiology, since mutations in SERCA1, SERCA2, and SPCA1 cause, respectively, Brody disease, Darier disease, and Hailey−Hailey disease.

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