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## Acidic calcium stores open for business: expanding the potential for intracellular Ca<sup>2+</sup> signaling

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### Abstract

Changes in cytosolic calcium concentration are crucial for a variety of cellular processes in all cells. It has long been appreciated that calcium is stored and released from intracellular calcium stores such as the endoplasmic reticulum. However, emerging evidence indicates that calcium is also dynamically regulated by a seemingly disparate collection of acidic organelles. Here, we review the defining features of these acidic calcium stores and highlight recent progress in understanding the mechanisms of uptake and release of calcium from these stores. We also examine the nature of calcium buffering within the stores and summarize the physiological and patho-physiological significance of these ubiquitous organelles in calcium signaling.

### Introduction

Changes in cytosolic calcium concentration constitute an evolutionarily conserved signaling pathway crucial for a huge number of physiological events [1]. Whilst in the resting cells, calcium concentration in the cytosol is maintained at low levels, typically 100 nM, the concentration can increase dramatically in response to a variety of environmental cues such as stressors and hormones [1]. These calcium signals display spectacular spatiotemporal complexity. Not surprisingly, deviant calcium signals are associated with cellular dysfunction and death [2]. Thus, understanding the mechanism by which calcium signals are generated is of up most importance in understanding how cells ultimately function correctly.

Dynamic changes in calcium have been extensively studied in animal cells. Calcium is derived from the extracellular space and/or intracellular calcium stores [3]. The endoplasmic reticulum is by far the best studied releasable calcium store possessing a well defined repertoire of calcium channels, pumps and buffers [4]. Calcium, however, is also stored in a variety of acidic organelles in both prokaryotic and eukaryotic cells. This was recognized as early as the 1970s using histochemical and electron microscopy techniques [5]. In this review, we describe the

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form of these acidic calcium stores, the mechanisms of calcium handling by these stores and the functional roles of these ubiquitous organelles in calcium signalling.

## Acidic calcium stores: Form and function

Here, we classify a number of organelles, which at first sight appear very different, as acidic calcium stores (Fig. 1). This classification is based on their acidic interior and the presence of calcium. Acidic calcium stores include acidocalcisomes, vacuoles, endosomes, lysosomes, lysosome-related organelles, secretory granules and the Golgi complex (Fig. 1). Calcium uptake into acidic calcium stores is mediated by either calcium pumps or exchangers (Box 1, Table 1). Acidic calcium stores also possess several calcium-permeable channels such as members of the transient receptor potential superfamily, the two-pore channels and IP<sub>3</sub>/ryanodine receptors in order to effect calcium release (Box 2, Table 2).

### Box 1

#### Mechanism of calcium uptake into acidic calcium stores

Calcium ATPases are pumps found in membranes of various organelles and the plasma membrane that transport calcium against its concentration gradient from the cytosol into the lumen or the extracellular space [78,79] (Figure 1). This process requires hydrolysis of ATP. In trypanosomes and *Toxoplasma*, a calcium ATPase related to the plasma membrane type calcium ATPase (PMCA) in mammals [78] is located in acidocalcisomes where it is responsible for filling of these stores with calcium [6,7]. ATPases of the same subtype have been described in the vacuoles of *Dictyostelium* [11], yeast [12] and plants [80]. Whether they are also present in the acidic calcium stores of animal cells is not known.

Sarco/endoplasmic reticulum ATPases (SERCAs), as their name implies, fill endoplasmic reticulum calcium stores with calcium [78]. These pumps however have also been implicated in the filling of acidic calcium stores such as the Golgi [81]. Additionally, in a number of secretory granules, such as large dense core granules of PC12 cells [37], insulin storage granules of mouse pancreatic beta-cells [82] and bovine adrenal chromaffin granules [35], Ca<sup>2+</sup> accumulation was shown to be ATP-dependent and sensitive to inhibitors commonly used against SERCA-type pumps (e.g. thapsigargin, cyclopiazonic acid). Results with human platelets also suggest that Ca<sup>2+</sup> uptake into acidic stores is driven at least in part by SERCA-type Ca<sup>2+</sup>-ATPase [83,84].

The third class of calcium pumps are the secretory pathway Ca<sup>2+</sup> ATPases (SPCAs) [79]. These proteins mediate Ca<sup>2+</sup> uptake in the Golgi [81] and certain secretory vesicles [36]. SPCA was first described in yeast and named Pmr1p [73]. In addition to calcium, it is also able to transport Mn<sup>2+</sup> and both ions are needed for many enzymatic reactions in the Golgi apparatus [81].

Finally, isolated lysosomes from neutrophils [85] and fibroblasts [86] have been reported to take up Ca<sup>2+</sup> and a Ca<sup>2+</sup>-ATPase activity has been measured in isolated lysosomes from rat liver [87,88]. Ca<sup>2+</sup>-ATPase activity has also been detected in synaptic vesicles [89]. In all cases however the molecular nature of the pump has not been identified.

The large concentration gradient of protons across the membranes of acidic calcium stores can be readily utilized to transport calcium into the vesicle lumen against its concentration gradient. This process is mediated by calcium hydrogen exchangers (CAXs) [90]. CAXs have been characterized in acidocalcisomes of protists [6] and in both yeast [91] and plant [92] vacuoles. Interestingly, although the genes encoding CAXs are present in the genomes of some animals (e.g. fish and amphibians), they are absent in mammalian genomes [90]. Nevertheless, calcium hydrogen exchange activity has been reported in isolated melanosomes from retinal pigment epithelial cells [93], large dense core granules of PC12

cells [37] and synaptic vesicles of sheep brain cortex [89]. One possibility to explain this apparent paradox is that, in mammals, calcium hydrogen exchange activity might not be mediated by a single protein but instead through the concerted activity of sodium hydrogen exchangers and sodium calcium exchangers, the net effect being the transport of calcium ions into the lumen and protons out of the lumen. Indeed, sodium calcium exchange activity has been detected in secretory granules [37,94] and at least one member of the sodium-calcium exchanger family NCKX5 is an intracellular protein present in the melanosomes and/or Golgi [71,95].

## Box 2

### Mechanisms of calcium release from acidic calcium stores

Transient receptor potential (TRP) channels are a large superfamily of distantly related cation channels involved in the transduction of sensory stimuli [41] (Figure II). In yeast, *yvc1* (TRPY1), has been identified as a calcium-permeable channel localized to the vacuole that can be activated by cytosolic calcium and membrane stretch [15,96]. TRP mucolipins (TRPML) are a subfamily of TRP channels that, together with the TRP polycystins, constitute the group II TRP channels [41]. TRPMLs are so-named based on the discovery that the gene encoding the founding member (TRPML1) in humans is mutated in the lysosomal storage disease mucopolipidosis IV [97]. Mammals possess genes encoding three TRPML proteins [97]. TRPML1 localizes to the late endosomes/lysosomes, TRML2 to recycling endosomes, late endosomes/lysosomes and the plasma membrane, and TRPML3 throughout the endolysosomal system and plasma membrane [97]. The channel properties of TRPMLs are subject to debate, although several studies suggest that they can function as calcium channels [97]. These channels show a wide tissue distribution in mammals, suggesting a general role for TRPMLs in regulating calcium fluxes from the endo-lysosomal system. The physiological trigger for calcium release by TRPMLs, however, remains to be established.

Two-pore channels (TPCs) are poorly characterized ion channels that were originally identified based on sequence similarity to voltage-sensitive sodium and calcium channels [16,98]. The latter two comprise four repeats, whereas TPCs are unique in that they possess only two of these repeats [16,98]. In plants, TPCs localize to the vacuole and are likely the molecular correlate of the slow vacuolar (SV) current [17]. SV channels are calcium permeable and activated by cytosolic calcium and positive membrane potentials [17]. Consistent with this mode of activation, plant TPCs possess a pair of putative EF-hands within the cytosolic loop, which likely bind to calcium, and a series of positively charged amino residues within the predicted fourth transmembrane regions of each repeat that are likely to serve as voltage sensors [16,17,98]. Little is known concerning the properties of the animal TPCs.

It is well established that mobilisation of endoplasmic reticulum calcium stores can be mediated via inositol trisphosphate and ryanodine receptors [1]. These calcium channels are gated by the calcium-mobilizing messengers inositol trisphosphate and cyclic ADP-ribose, respectively, and share many structural and functional similarities [1]. Although their predominant location is within the membranes of the endoplasmic reticulum, these channels can also be located within additional subcellular locales, including acidic calcium stores. *Paramecium* possesses an expanded family of inositol trisphosphate receptors and one of the isoforms localizes to the contractile vacuole [99]. Additionally, both inositol trisphosphate and cyclic ADP-ribose have both been reported to release calcium from the plant vacuole [100], but genes encoding receptors for inositol trisphosphate and ryanodine are apparently absent from plant and yeast genomes. In animal cells, inositol trisphosphate

receptors have been localized to the acrosome from sperm [101], and inositol trisphosphate-induced calcium release has been demonstrated from secretory vesicles isolated from the adrenal medulla and several other secretory cell types [34]. Cyclic ADP-ribose has been reported to release calcium from zymogen granules or a closely associated acidic organelle from exocrine pancreatic acinar cells [102] and dense core vesicles in a clonal pancreatic beta cell line [36]. Additionally, caffeine (a pharmacological activator of ryanodine receptors) was shown to mediate calcium release from isolated melanosomes [93]. Clear evidence has also been obtained for functional inositol trisphosphate receptors on the Golgi complex [39]. Thus, receptors for inositol trisphosphate and ryanodine likely mediate calcium release from acidic calcium stores as well as from the endoplasmic reticulum.

### Acidocalcisomes

Based on its presence in prokaryotes, the earliest calcium-containing acidic compartment that appeared during evolution was probably the acidocalcisome [6]. The acidocalcisome was first identified in bacteria and called the volutin granule [6]. Recent work in *Agrobacterium tumefaciens* and *Rhodospirillum rubrum* has demonstrated that acidocalcisomes are membrane bounded and express a vacuolar proton pyrophosphatase (V-PPase) that drives acidification [6]. Acidocalcisomes in both prokaryotes and eukaryotes such as trypanosomes are characterized by their electron-density in the absence of staining and are easily visualized by direct transmission electron microscopy (Fig. 2). Like other acidic calcium stores, some acidocalcisomes possess a vacuolar proton ATPase (V-ATPase) in place or in addition to a V-PPase to maintain an acidic pH typically 5–5.5 [6]. Acidocalcisomes contain high concentrations of calcium, at millimolar levels, and serve several functions, including storage of cations and phosphorus, polyphosphate metabolism, maintenance of intracellular pH homeostasis and osmoregulation [6]. Silencing of the *TbPMCI* gene in *Trypanosoma brucei*, which encodes a  $\text{Ca}^{2+}$ -ATPase pump localized to the acidocalcisome, results in reduced mobilizable calcium from these stores and is associated with impaired growth [7]. Deletion of the corresponding gene in *Toxoplasma gondii* (*TgAI*) also affects growth and has dramatic effects on invasion and virulence [8]. These findings underscore the physiological importance of calcium uptake into acidocalcisomes for their normal functioning. Calcium permeable channels located on acidocalcisomes however have yet to be identified.

### Vacuoles

The contractile vacuole present in protists and slime molds is closely related to the acidocalcisome [6]. Both organelles are rich in calcium and polyphosphate and, in *Trypanosoma cruzi*, they are functionally linked as evidenced by the translocation of an aquaporin channel from acidocalcisomes to the contractile vacuole during regulatory volume decrease after hyposmotic stress [6]. Yeast vacuoles and the storage and lytic vacuoles in plants also share many similarities with acidocalcisomes, such as the presence of several pumps (V-ATPase, V-PPase,  $\text{Ca}^{2+}$ -ATPase), exchangers ( $\text{Na}^+/\text{H}^+$ ,  $\text{Ca}^{2+}/\text{H}^+$ ), and aquaporin [6]. Importantly vacuoles in both yeast [9] and plants [10] are the major site of intracellular  $\text{Ca}^{2+}$  storage. In support, deletion of vacuolar calcium ATPase pumps in both *Dictyostelium* [11] and yeast [12] renders the cells less tolerant to high extracellular calcium concentrations. Interestingly, transcripts for ACA4, a vacuolar  $\text{Ca}^{2+}$ -ATPase from *Arabidopsis*, are present at increased levels during salt stress and provide salt tolerance when heterologously expressed in yeast [13]. Salt stress is associated with aberrant calcium signaling [14]. Thus, uptake of calcium into the vacuole might serve to temper these changes.

Genetic manipulation of the vacuolar channels TRPY1 in yeast and TPCs in plants has provided much insight into the role of vacuolar calcium release in the regulation of cell function. Both channels have been implicated in mediating calcium signals in response to hyperosmotic stress

[15,16]. Several additional roles for plant TPCs have been identified including germination and stomatal movement [17]. Release of acidic calcium stores in yeast and plants is therefore vital for a variety of cellular processes.

### Endosomes and lysosomes

The endolysosomal system of eukaryotes comprises many acidic organelles that contain variable amounts of calcium. Endosomes, once formed at the plasma membrane, become gradually acidified to a pH of ~6 as they mature. This change in pH is important for endosome function. For example it promotes the release of ligands from internalized receptors during receptor-mediated endocytosis. Given their origin, endosomes are expected to have the same free  $\text{Ca}^{2+}$  concentration within their interior as the extracellular fluid (~ 1 mM) after forming; however, there are few studies that have directly measured endosomal calcium levels. In 3T3 Swiss fibroblasts, calcium is apparently rapidly released from newly formed endosomes such that the luminal free concentration falls to ~ 3  $\mu\text{M}$  within 20 minutes of formation [18]. Using similar methods, the concentration of calcium in enlarged endosome-like structures formed in pancreatic acinar cells stimulated with supramaximal concentrations of the brain-gut peptide cholecystokinin was recently reported to be higher at ~ 40  $\mu\text{M}$  [19] but at the lower end of the free calcium concentration measured in the endoplasmic reticulum (60 – 400  $\mu\text{M}$ ) [20]. Nevertheless, the luminal endosomal calcium concentration is still at least an order of magnitude higher than resting cytosolic calcium levels. Thus, endosomes can also be considered acidic calcium stores, that are clearly mobile perhaps fuelling more restricted local changes in cytosolic calcium concentration.

Lysosomes are ubiquitous organelles that are highly acidic (pH 4–5) and which are responsible for the recycling of cellular constituents delivered by endocytic and autophagic routes. In addition to this well established role, there is currently a growing appreciation of these organelles as calcium stores. The presence of releasable  $\text{Ca}^{2+}$  in lysosomes has been demonstrated based on experiments using glycyl-L-phenylalanine-naphthylamide (GPN). GPN is a lysosome-disrupting cathepsin-C substrate that was originally used to distinguish lysosomes, which contain cathepsin C, from prelysosomal endocytic vacuoles, which do not [21]. Hydrolysis of GPN by cathepsin C induces osmotic swelling of the lysosome and release of its content, including  $\text{Ca}^{2+}$ , into the cytoplasm. Indeed, GPN induces increases in cytosolic calcium concentration when applied to a variety of different cells including MDCK cells [22]. Figure 3 highlights the presence of GPN-sensitive calcium stores in neurons. Direct measurement of luminal calcium concentration of lysosomes is difficult given the harsh acidic and proteolytic environment. Nevertheless, carefully controlled experiments in macrophages [23] and more recently fibroblasts [24] using internalized fluorescent indicators indicate that the average free calcium concentration in lysosomes is approximately 500  $\mu\text{M}$  and thus comparable to the concentration within the endoplasmic reticulum [20].

A multitude of hormones and neurotransmitters evoke changes in cytosolic calcium through production of calcium-mobilizing messengers that, in turn, mobilize intracellular calcium stores [1]. Although this process is generally ascribed to inositol trisphosphate or cyclic ADP-ribose which both mobilize endoplasmic reticulum calcium stores, much evidence indicates an additional critical role for NAADP (Box 3). Importantly, as first shown in the sea urchin egg [25], NAADP-mediated calcium signals are blocked by GPN indicating that NAADP mobilizes calcium from lysosomes or lysosome-like compartments [26]. NAADP is also likely to release calcium from endosomes [27]. Thus, the endo-lysosomal system serves as a *bonafide* mobilizable calcium store.

**Box 3****NAADP**

NAADP is the newest and most potent of the calcium-mobilizing messengers [26] (Figure III). In sea urchin eggs, where the calcium-mobilizing ability of NAADP was first discovered [103], the pharmacology of NAADP-induced calcium release and the biochemical properties of the binding protein are very distinct from established endoplasmic reticulum calcium channels, indicative of a novel channel [103,104]. Moreover, Churchill and colleagues identified the target calcium stores as lysosome-like by showing selective block of NAADP-mediated calcium signals by the lysomotropic agent GPN and also by bafilomycin-A<sub>1</sub> [25]. The latter is an inhibitor of V-type ATPases, which by collapsing the pH gradient across the membrane of the acidic store, probably prevents calcium uptake. Thus, unlike inositol trisphosphate and cyclic ADP-ribose, which generally mobilize endoplasmic reticulum calcium stores through well-characterized channels (but see Box 2), NAADP appears highly unusual in activating channels located on acidic calcium stores. These include endosomes [27], lysosomes [105], lysosome-related organelles [25] and secretory granules [40]. Although, the molecular identity of the channel is subject to debate, several extracellular cues that elevate cytosolic calcium levels do so through raising cellular NAADP levels. These range from egg jelly acting on sea urchin sperm cells [106] to the neurotransmitter glutamate acting on mammalian brain slices [107]. Intriguingly, NAADP-sensitive calcium channels rarely operate in isolation. Instead, current evidence suggests that, upon generation, NAADP initially evokes a relatively small trigger [108] release of calcium from acidic calcium stores, which in turn sensitizes neighbouring channels on the endoplasmic reticulum to mediate larger calcium signals by calcium-induced calcium release [26] (Fig. III). Importantly, this chatter [109] between NAADP-sensitive acidic calcium stores and other calcium sources has been implicated in a variety of cellular processes ranging from fertilization [106,110] to neuronal differentiation [111]. At present, this pathway represents the best-characterized pathway for calcium release from acidic calcium stores in animal cells. NAADP signaling has also been described in plants [112] but not in micro-organisms. Gross, in a recent insightful review [113], has proposed that functional cross-talk between acidic calcium stores and the endoplasmic reticulum may underlie differentiation in *Dictyostelium*. It is tempting to speculate that this may involve NAADP particularly given the presence of the two-pore channel, a strong candidate NAADP target (see main text) in this organism.

**Lysosome-related organelles**

Lysosome-related organelles, often referred to as secretory lysosomes, are a heterogeneous group of organelles that can be defined as sharing some physiological features with lysosomes [28,29]. Such organelles include melanosomes, lytic granules of lymphocytes, major histocompatibility complex class II compartments of antigen-presenting cells (dendritic cells, B lymphocytes and macrophages), platelet dense granules, basophilic granules, neutrophil azurophil granules [28], and, possibly, others such as platelet alpha granules, lamellar bodies of the lung, osteoclast granules, pigment granules of *Drosophila*, the fat storage organelle of the nematode *Caenorhabditis elegans*, Weibel-Palade bodies of endothelial cells, acrosomes from sperm and inhibitory lysosomes from dendritic cells [29]. Many lysosome-related organelles are acidic and rich in calcium. Platelet dense granules for example maintain a pH of ~5.4 and a total calcium concentration of 2.2 M [30]. These organelles are especially interesting acidic stores because they resemble acidocalcisomes in that they contain polyphosphate and are electron dense when examined by direct transmission electron microscopy without staining [31]. In addition, acidocalcisomes and platelet dense granules appear to share the system for targeting of their membrane proteins through adaptor protein 3

[32]. This led to the proposal that acidocalcisomes are conserved from bacteria to man and that other acidocalcisome-like organelles are likely present in different organisms [6]. Indeed, acidocalcisome-like organelles have also been found recently in insect [33] and sea urchin eggs (Ramos, I.B. et al., unpublished observation).

### Secretory vesicles and the Golgi complex

Finally, the acidic compartments of the secretory pathway also contain substantial levels of calcium.  $\text{Ca}^{2+}$  is highly abundant in secretory granules of many secretory cell types and the related synaptic vesicles of neurons. Remarkably, some secretory granules such as those in adrenal chromaffin cells contain 20–40 mM total calcium [34] and are therefore a major store of calcium. Free  $\text{Ca}^{2+}$  in secretory granules has been measured between 50–100  $\mu\text{M}$  [35]. Secretory granules in both pancreatic beta cells [36] and adrenal chromaffin cells [37] take up calcium upon secretagogue stimulation. This process might serve to moderate cytosolic calcium similar to uptake of  $\text{Ca}^{2+}$  by vacuoles (see above). Thus, like the endoplasmic reticulum, acidic calcium stores may function as calcium “sinks” as well as calcium sources.

Notably, total calcium concentration in the Golgi has been estimated to be more than 10 mM in neurosecretory PC12 cells [38]. The free  $\text{Ca}^{2+}$  is measured between 300  $\mu\text{M}$  to 1.4 mM in HeLa cells [39] and therefore similar, if not higher than the concentration of calcium within the endoplasmic reticulum. Thus, although less acidic than the other organelles discussed (pH ~ 6.6), the Golgi apparatus can also be considered an acidic calcium store.

The possible presence of inositol trisphosphate/ryanodine receptors on secretory granules and the Golgi complex (Box 2) suggests that mobilisation of these stores might contribute to cytosolic calcium signals upon receptor-mediated production of inositol trisphosphate and cyclic ADP-ribose or through calcium-induced calcium release stimulated by other routes. NAADP may also be involved given the presence of the target channels on secretory granules [40]. The localization of calcium release channels on secretory organelles is particularly interesting as generation of local calcium signals close to the calcium-dependent fusion machinery might in part drive secretion. Alternatively, it is possible that calcium within these organelles may serve functional roles in the extracellular space upon its secretion.

### Mechanisms of calcium release from acidic calcium stores: New players

TRPMs and TRPVs belong to the group 1 TRP subfamily [41]. TRPM2 is an unusual protein in that it is a so-called chanzyme, possessing both ion channel and enzymatic (ADPR pyrophosphatase) activity [41]. Electrophysiological analysis indicates that TRPM2 is non-selective cation channel permeable to calcium that is activated by cytosolic ADP-ribose and a variety of other factors including hydrogen peroxide [41]. Like other TRPMs it is localized to the plasma membrane to mediate calcium influx [41]. However, a recent intriguing report has localized the protein to the lysosome, where it functions as a calcium release channel mediating cell death in response to oxidative stress [42]. The reported sensitivity of TRPM2 to NAADP [43] might also suggest an involvement in NAADP-mediated mobilization of lysosomal calcium stores [44]. TRPV2, in common with several other TRPV members, is activated by heat [41]. Proteomic analysis has revealed the presence of TRPV2 on early endosomes [45]. Indeed a channel with the biophysical properties similar to those of TRPV2 has been identified electrophysiologically on enlarged endosomes [46]. Thus, TRPV2, like TRPM2, shows a dual distribution on the cell surface and within acidic calcium stores and might therefore mediate both calcium influx and calcium release. Alternatively, the intracellular location could reflect trafficking of these channels to and from the plasma membrane.

Two-pore channels, as discussed previously (Box 2), have been most extensively studied in plants. In a series of recent independent studies, direct molecular evidence has been obtained

indicating that, in animals, the two-pore channel is the target for NAADP. Human TPC1 was shown to localize to both endosomes and lysosomes [47,48], whereas human and rodent TPC2 were shown to be predominantly lysosomal [47–49], consistent with the vacuolar location of plant TPCs [17]. Importantly, over-expression of TPC1 and TPC2 enhanced NAADP-mediated calcium signals [47–49]. Conversely, siRNA-mediated knockdown of TPC1 attenuated NAADP-mediated calcium signals in human SKBR3 cells [47]. Additionally, NAADP-dependent plasma membrane currents in pancreatic beta cells from TPC2 knockout mice were absent [48]. Finally, mutation of a single conserved residue within a putative pore region was sufficient to prevent calcium release by TPC1 and revealed dominant-negative activity [47]. Taken together, these studies identify this ubiquitous yet poorly characterized family of ion channels as credible targets for NAADP, providing a molecular basis for calcium release from acidic stores by this messenger. Like TRP channels (Box 2), TPCs are widely distributed in mammalian cells [47–49], indicating that they too might play a general role in the mobilisation of acidic calcium stores.

Comparative genomic analysis indicates the presence of two-pore channels throughout the animal kingdom. However, these channels are notably absent in certain organisms including flies (*D. melanogaster*) and nematode worms (*C. elegans*). Most deuterostomes however, possess three genes. In the sea urchin, a basal deuterostome and extensively used model system for the study of NAADP-mediated calcium signaling, all three isoforms have been cloned and characterized [50]. Like mammalian TPCs, sea urchin TPCs localize to acidic organelles and enhance NAADP-mediated calcium signals when overexpressed [50]. Intriguingly, although the full TPC gene complement is found in *Laurasiatherian* mammals (such as dogs and cows), the TPC3 gene appears to be absent from certain mammals (such as rats and humans) that belong to the closely related sister group, *Euarchontoglires*. Moreover, evidence for a *TPC3* pseudogene has been provided in the primate lineage [50]. Thus, degeneration of TPC3 is a rare, relatively recent and on-going event likely indicative of relaxed selection pressure. Further comparative genomics and physiology will no doubt provide clues to the functional role of TPC3.

P2X receptors are ligand-gated non-selective cation channels regulated by extracellular ATP. In *Dictyostelium*, a P2X receptor homologue has been identified and localized to the contractile vacuole where it was shown to be involved in osmoregulation [51]. How the channel with its ligand binding site facing the vacuole lumen in this location would access ATP is not clear but it is possible that ATP is imported from the cytosol where it is present at millimolar levels. Recent studies in mammalian cells also place rat P2X4 receptors within lysosomes where they resist degradation by virtue of their N-linked glycans [52]. Prior stimulation of cells with agents that promote lysosome secretion was shown to increase subsequent channel activity at the plasma membrane. Thus, in this case the lysosomal location of a calcium permeable channel probably reflects its transit to its ultimate destination. However, that lysosomes contain ATP might suggest hitherto unsuspected intracellular roles, such as in phagocytosis [52], for this traditionally viewed cell surface channel.

## Mechanisms of calcium buffering within acidic calcium stores

The available information concerning how calcium is buffered in acidic compartments is at present limited. Most of the calcium present in acidic stores is likely bound to polyanionic matrixes. Notably, polyphosphate is present in many acidic calcium stores such as acidocalcisomes of different organisms [6], the yeast vacuole [53] and probably lysosomes [54] and lysosome-related organelles [31]. Polyphosphate is a linear polymer of inorganic phosphate moieties, from a few to several hundred, linked by high-energy phosphoanhydride bonds [55]. It is capable of binding calcium and other cations and basic organic molecules, such as basic amino acids, in a semicrystalline state. This is revealed by magic-angle spinning



NMR detection of solid-state condensed phosphate and the high electron density of acidocalcisomes *in situ* [6]. Evidence for association of calcium with polyphosphate comes from experiments showing that alkalization of acidocalcisomes led to  $\text{Ca}^{2+}$  release and hydrolysis of polyphosphate in a number of cells [6]. Polyphosphate might therefore represent the major  $\text{Ca}^{2+}$  buffer present in acidic calcium stores.

Oxalates likely play a similar  $\text{Ca}^{2+}$  buffering role in plants. Oxalates are abundant in the plant vacuole where they precipitate calcium, forming crystals [56]. In animal cells, other polyanions such as heparin present in basophilic granules [57], or mucin present in mucinogen granules of Goblet cells [58], might also buffer intragranular calcium, but there is little information about their physiological role. Finally, luminal proteins might also contribute to calcium buffering in lysosome-related organelles and secretory granules. Melanosomes, for example are rich in melanin, a  $\text{Ca}^{2+}$ -binding protein [59]. It has been found that melanocytes richest in melanin are able to accumulate more  $\text{Ca}^{2+}$  and buffer cytosolic  $\text{Ca}^{2+}$  changes better than those possessing less melanin [59]. The granins present in neurosecretory granules are probably the best-characterized proteins present in acidic calcium stores with respect to their  $\text{Ca}^{2+}$ -binding properties. They bind to calcium with low affinity and high capacity, a property that allow them to form aggregates at low pH in the presence of calcium [60]. The presence of buffering mechanisms within acidic calcium stores is consistent with their role as mobilizable calcium stores.

## Dysfunction of acidic calcium stores

Many diseases result from deregulated calcium signalling. With appropriate means to fill with, buffer and release calcium, it is perhaps not surprising that dysfunction of acidic calcium stores is also linked with certain diseases.

The endolysosomal system and the secretory pathway are highly dynamic structures in which membrane fusion between vesicles is crucial for correct functioning. The role of calcium in stimulating membrane fusion is well established in the process of regulated secretion. Calcium might also play crucial roles in so-called constitutive fusion events, which are traditionally thought of as being calcium independent. Fusion *in vitro* of yeast vacuoles [61], early endosomes from rat PC12 cells [62], late-endosome-lysosomes from rat liver [63] and Golgi vesicles from rat brain [64] is inhibited by chelating extra-vesicular calcium. Chelating luminal calcium also blocks fusion [62] suggesting that calcium release from the acidic calcium store is required for fusion. Interestingly, in all of the above cases, inhibition of fusion is readily observed with the calcium chelator BAPTA but not with EGTA. Although both chelators bind to calcium with similar affinity, the former does so more rapidly [65]. Such differential sensitivity to BAPTA and EGTA suggests that the calcium signal must be localized very close to the putative sensor thus evading buffering by the slower chelator. The nature of the calcium channel(s) responsible for this local release of calcium however is not known. A strong candidate is TRPML1 as cells derived from mucopolidosis IV sufferers (who possess a defective *TRPML1* gene) display disrupted trafficking within the endo-lysosomal system [66], consistent with impaired fusion. A similar phenotype is observed in worms deficient in the TRPML homologue, CUP5 [67]. Direct evidence for a role for TRPML in the release of calcium that regulates membrane fusion, however, is currently lacking.

As discussed above, the two-pore channels have recently emerged as the likely targets for NAADP in animal cells [47–50]. As research into animal TPCs is only in its infancy, functional roles for this protein downstream of calcium signalling have yet to be identified. Coding variants in TPC2 have however been associated with hair pigmentation traits [68]. These findings potentially link pigmentation to NAADP-mediated calcium signals. Interestingly, a gain-of-function mutation in the endo-lysosomal calcium channel TRPML3 that causes the

mouse Varitint-Waddler phenotype also affects pigmentation (coat colour) [69] consistent with a role for lysosomal calcium release in regulating pigmentation. Indeed, pigmentation defects are common in several lysosomal storage diseases [70], perhaps as a consequence of altered calcium signalling through lysosomes or lysosome-related organelles secondary to dysfunction of the lysosomal target [24]. Notably, a coding variant in *NCKX5*, a melanosomal/Golgi calcium exchanger also correlates with changes in skin pigmentation in fish and humans [71].

Finally, altered calcium signaling through acidic calcium stores in the pancreas and skin have also been linked to disease. Acute pancreatitis is characterized by inappropriate activation of proteases in pancreatic acinar cells. This can be induced by excessive alcohol consumption. Recent studies have shown that fatty acid ethyl esters (alcohol metabolites) activate inositol trisphosphate receptors on both acidic stores within the secretory pole and in the ER [72]. Only the former however resulted in intracellular protease activation suggesting that their mobilisation might specifically contribute to disease progression [72]. Deletion of the *PMR1* gene in yeast, which encodes a calcium ATPase localized to the Golgi [73], impairs Golgi functions such as proteolytic processing and protein glycosylation [74] and is associated with elevated cytosolic calcium levels [75]. Notably, mutation of the orthologous gene in humans (*SPCA1*) results in the blistering skin disorder Hailey–Hailey disease [76]. Cytosolic calcium levels are also elevated in this disease [76] and Golgi luminal calcium concentration reduced [77]. These findings underscore the importance of calcium uptake into this acidic store for both proper functioning of the organelle and in the maintenance of cytosolic calcium homeostasis.

## Future perspectives

We have argued that acidic calcium stores are present in probably all organisms. They differ significantly in their form and can coexist in the same cell. It is clear that acidic calcium stores can accumulate vast levels of calcium, although measurements of the free (mobilizable) concentration of calcium are scant and should be the focus of future studies. Much progress has been made in identifying the calcium pumps/exchangers and calcium-permeable channels present on these stores with clear physiological roles for calcium uptake into acidocalcisomes, both calcium uptake and  $\text{Ca}^{2+}$  release from the vacuoles of yeast and plants and for NAADP-mediated calcium release from lysosomes and lysosome-related organelles in animals. However there are still gaps in our knowledge with respect to the calcium channel complement of acidocalcisomes, the uptake machinery of the endo-lysosomal system and in general how calcium is buffered within these organelles. A comprehensive molecular description of the calcium signaling “tool kit” for these organelles is required. This is probably forthcoming in the near future as advances in organelle proteomics continue but one needs to consider that the presence of calcium channels in an acidic calcium store need not necessarily equate to a functional role at this location and could, in some cases, reflect their trafficking. Future studies might also consider the issue whether the presence of calcium within a given acidic store may regulate luminal processes perhaps dynamically as calcium is sequestered and released. All of these questions are deserving of answer as we now begin to associate defective calcium handling by acidic calcium stores with disease.

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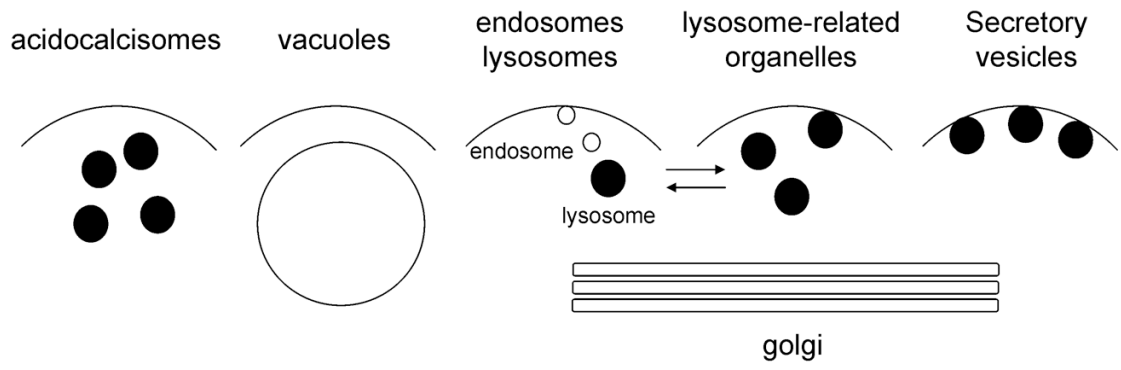
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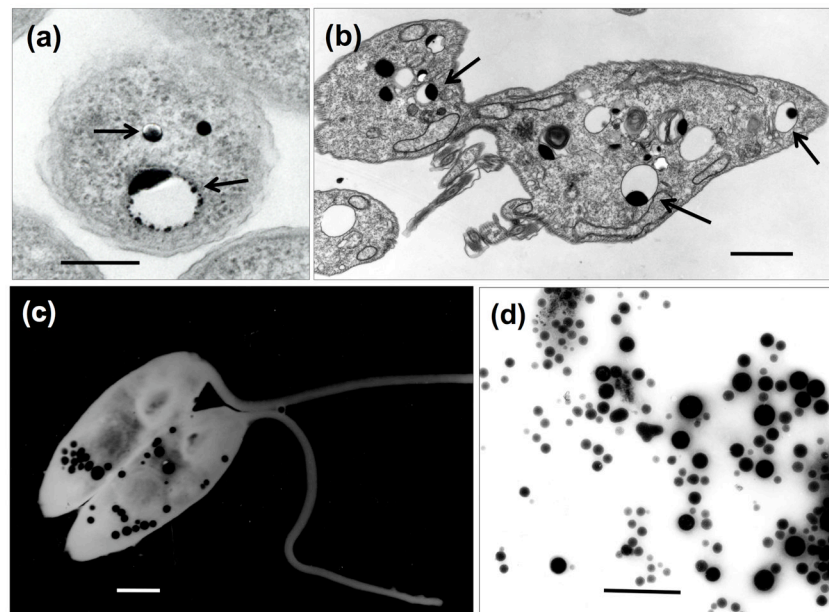
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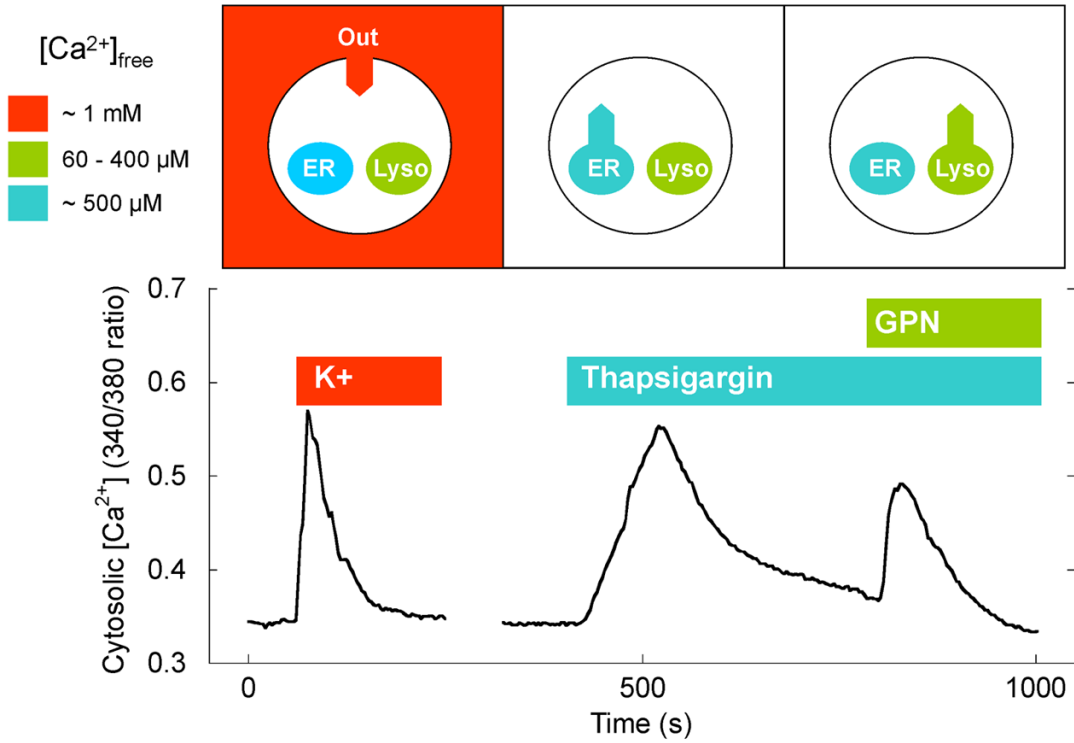


**Figure 1. The acidic calcium stores**  
Schematic representation of acidic organelles that contain high concentrations of calcium.



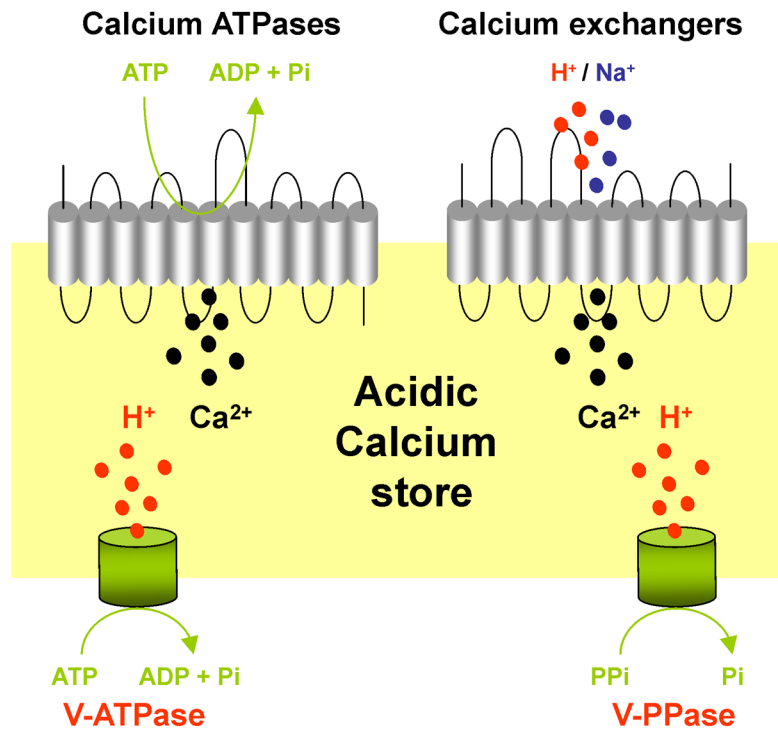
**Figure 2. Acidocalcisomes in whole cells and subcellular fractions**

(a), (b), ultrathin sections of *Agrobacterium tumefaciens* (a) or *Trypanosoma brucei* procyclic forms (b) showing the acidocalcisomes as empty vesicles with electron-dense inclusions (arrows). (c), (d), visualization of acidocalcisomes in whole unfixed *Herpetomonas angusteri* and *Trypanosoma cruzi* acidocalcisome fraction obtained by iodixanol centrifugation [6] allowed to adhere to Formvar- and carbon-coated grids and then observed by direct transmission electron microscopy (using an energy filter in (c)). Acidocalcisomes (black granules) appear disperse in the cytoplasm (c) or fraction (d). Scale bars: (a), 0.2  $\mu\text{m}$ ; (b), 2  $\mu\text{m}$ ; (c), 0.5  $\mu\text{m}$ ; (d), 1  $\mu\text{m}$ . (a) was taken by Manfred Seufferheld. (c) is reproduced with permission from ref. [114].



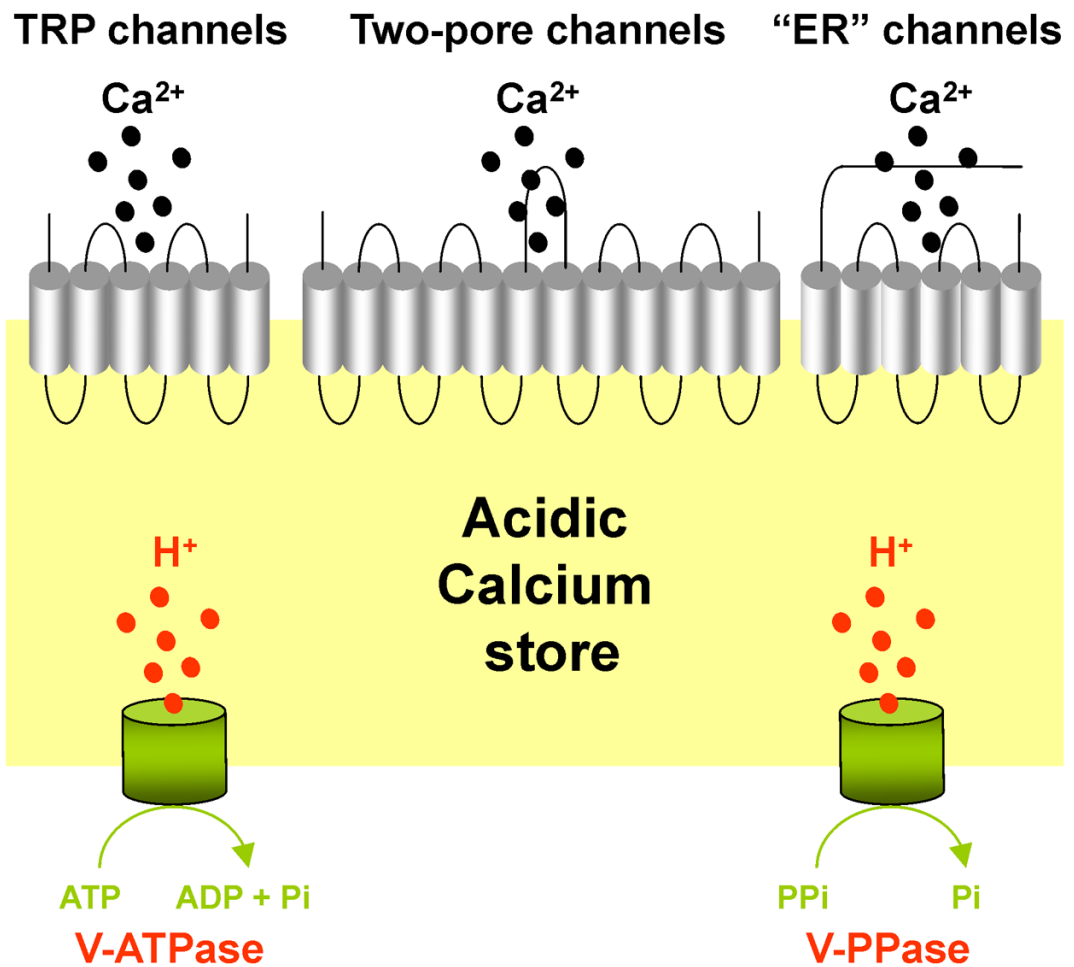
**Figure 3. Lysosomal calcium stores in neurons**

Top, Schematic representation of a cell depicting high levels of calcium on the outside of the cell (red), within endoplasmic reticulum calcium stores (blue) and within lysosomal calcium stores (green). The concentration of free calcium in these compartments ( $[Ca^{2+}]_{free}$ ) is listed. The bottom panel shows measurements of cytosolic calcium levels in hippocampal neurons upon depolarization with high  $K^+$  (to stimulate calcium influx from the extracellular space). The cells were then sequentially stimulated (in the absence of external calcium) with thapsigargin (to stimulate depletion of ER calcium stores) and GPN (to lyse lysosomal calcium stores). All three maneuvers raise the cytosolic calcium concentration. The data in the bottom panel was obtained by George Dickinson.



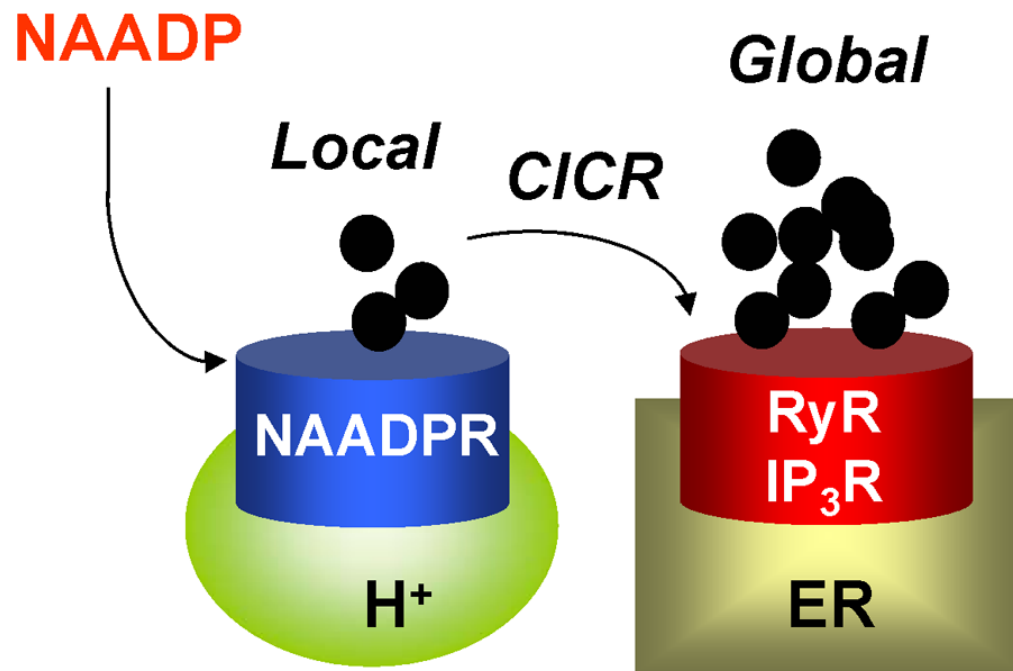
**Box 1 Figure I. Calcium transport in to acidic calcium stores**

Schematic of an acidic calcium store depicting calcium pumps and exchangers that mediate calcium uptake (top) and V-ATPases and V-PPases responsible for acidification (bottom).



**Box 2 Figure II. Calcium release from acidic calcium stores**

Schematic of an acidic calcium store depicting calcium-permeable channels (top) which mediate calcium release (top) and V-ATPases and V-PPases responsible for acidification (bottom).



**Box 3 Figure III. NAADP-mediated channel “chatter”**

Schematic showing proposed mechanism of action of NAADP starting with the generation of a local calcium signal from an acidic calcium store and subsequent amplification by the ER resulting in a global calcium signal.

Table 1

Ca<sup>2+</sup> transport into acidic stores

Calcium pump/exchanger	General characteristics	Associated Human Genetic Diseases	Acidic store
PMCA-type Ca <sup>2+</sup> -ATPase (subgroup IIB)	P-type, 10 TMD Ca <sup>2+</sup> transport in exchange for H <sup>+</sup> One Ca <sup>2+</sup> binding site	Hereditary deafness (PMCA2)	Acidocalcisomes [6] Yeast vacuole [12] <i>Dictyostelium</i> contractile vacuole [11] Plant vacuole [80]
SERCA-type Ca <sup>2+</sup> -ATPase (subgroup IIA)	P-type, 10 TMD Ca <sup>2+</sup> transport in exchange for H <sup>+</sup> Two Ca <sup>2+</sup> binding sites Specific inhibition by thapsigargin and cyclopiazonic acid	Brody's disease (SERCA1) Darier-White's disease (SERCA2)	Lysosome-related organelles [83,84] Secretory granules [35,37,82] Golgi apparatus [81]
SPCA-type Ca <sup>2+</sup> -ATPase (subgroup IIA)	P-type, 10 TMD Ca <sup>2+</sup> and Mn <sup>2+</sup> transport One Ca <sup>2+</sup> /Mn <sup>2+</sup> binding site	Hailey-Hailey's disease (SPCA1)	Secretory granules [36] Golgi apparatus [81]
Unidentified Ca <sup>2+</sup> -ATPase	P-type, ND Ca <sup>2+</sup> transport	ND	Lysosomes [86-88] Synaptic vesicles [89]
Ca <sup>2+</sup> /H <sup>+</sup> exchanger (CAX)	11 TMD (two "half-proteins" separated by an "acidic motif") Reversible	ND	Acidocalcisomes [6] Yeast vacuole [91] Plant vacuole [92] Melanosomes <sup>#</sup> [93] Secretory granules <sup>#</sup> [37] Synaptic vesicles <sup>#</sup> [89]
Na <sup>+</sup> /Ca <sup>2+</sup> exchanger (NCX)	11 TMD, Ca <sup>2+</sup> exchange for Na <sup>+</sup> Reversible	ND	Secretory granules <sup>#</sup> [37,94] Melanosomes (NCKX5) [71]

<sup>#</sup>The molecular identity of the exchanger is unknown. ND, not determined; P-type: characterized by the formation of a phosphorylated enzyme intermediate, and inhibited by vanadate; TMD, transmembrane domains; NCKX5, potassium-dependent Na<sup>2+</sup>/Ca<sup>2+</sup> exchanger

**Table 2**Ca<sup>2+</sup> transport out of acidic stores

Calcium permeable channel	General characteristics	Associated human genetic diseases	Acidic store
TRP channels (TRPML1, TRPML2, TRPML3, TRPM2, TRPV2)	6 TMD	Mucopolidosis IV (TRPML1)	Yeast vacuole [15] Endosomes [46,97] Lysosomes [42,97]
Two-pore channels	12 TMD	ND	Plant vacuole [17] Endosomes [47,48] Lysosomes [47–49]
Inositol trisphosphate/Ryanodine receptors	6 TMD	Congenital myopathies, Malignant hyperthermia (RYR1) Arrhythmias (RYR2)	<i>Paramecium</i> contractile vacuole [99] Plant vacuole [100] Lysosome-related organelles [93, 101] Golgi complex [39] Secretory granules [34]

ND, not determined; TMD, transmembrane domains; TRPML, TRP mucolipins; TRPM, TRP melastatins; TRPV, TRP vallinoid