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PMCA – Plasma Membrane Ca^{2+}-ATPase

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1. General Information

The plasma membrane Ca^{2+}-ATPase (PMCA) is an ATP-driven Ca^{2+} pump ubiquitously expressed in the plasma membrane of all eukaryotic cells. The PMCA is the major Ca^{2+} efflux pathway in non-excitable cells, such as pancreatic acinar cells, where the Na^{+}/Ca^{2+}-exchanger (NCX) is either not expressed or has minimal functional role. Even in cells where NCX is expressed abundantly, PMCA is critical for maintaining cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) below 300 nM (~100 nM), due to its high affinity for Ca^{2+} (Kd, ~0.2 µM) (22, 23, 109).

Compared to other components of the Ca^{2+} signalling machinery the PMCA has received very little attention. This is because for many years the PMCA was thought to only have a minor house-keeping role in maintaining low resting $[Ca^{2+}]_i$ with very little role in regulating dynamic Ca^{2+} signalling. However, in recent years the importance of PMCA in the spatiotemporal shaping of cytosolic Ca^{2+} signalling has steadily increased. For example, time to activate appropriate stress response pathways or even initiate apoptosis, which is generally protective. However, even if other Ca^{2+} clearance pathways are active, if PMCA is inhibited then the Ca^{2+} essentially has nowhere else to go, inevitably leading to an irreversible

PMCA exhibits memory of past $[Ca^{2+}]_i$ increases (25), suggesting an important role in regulating the frequency of Ca^{2+} oscillations. Moreover, the different PMCA isoforms, and numerous splice variants of PMCA, can be differentially expressed in specific regions of cells and can also be differentially regulated by a sophisticated repertoire of additional signalling pathways (22, 23).

Nevertheless, despite this emerging role of the PMCA in dynamic Ca^{2+} signalling, the importance of the house-keeping role of the PMCA must not be underestimated, especially when one considers how important maintaining low resting $[Ca^{2+}]_i$ is for cell survival during pancreatic diseases such as pancreatitis, pancreatic cancer and diabetes. PMCA can be regarded as the last gatekeeper for the maintenance of low resting $[Ca^{2+}]_i$; even if all other Ca^{2+} clearance pathways are inhibited, if the PMCA is maintained or protected, cytosolic Ca^{2+} will nearly always recover giving the cell Ca^{2+} overload and the consequent necrotic cell death.

Structural features of the PMCA give rise to the functional diversity

PMCA is encoded by four separate genes (PMCA1-4) and numerous

splice variants that give rise to specific tissue distribution, cellular localisation and functional diversity (76, 108). PMCA1 and PMCA4 are ubiquitously expressed whereas PMCA2 and PMCA3 have a more tissue specific expression and tend to be more abundant in excitable cells (49). Structurally, PMCA consists of 10 transmembrane domains, 2 cytosolic loops with both N- and C-terminal cytosolic tails (see Figure 1) (76, 108). The N-terminal tail exhibits the greatest diversity between the different isoforms and has been used to generate isoform-specific antibodies (107). The only functional feature of the N-terminal domain is the recently discovered 14-3-3-binding domain which has been shown to be inhibitory (71, 97).

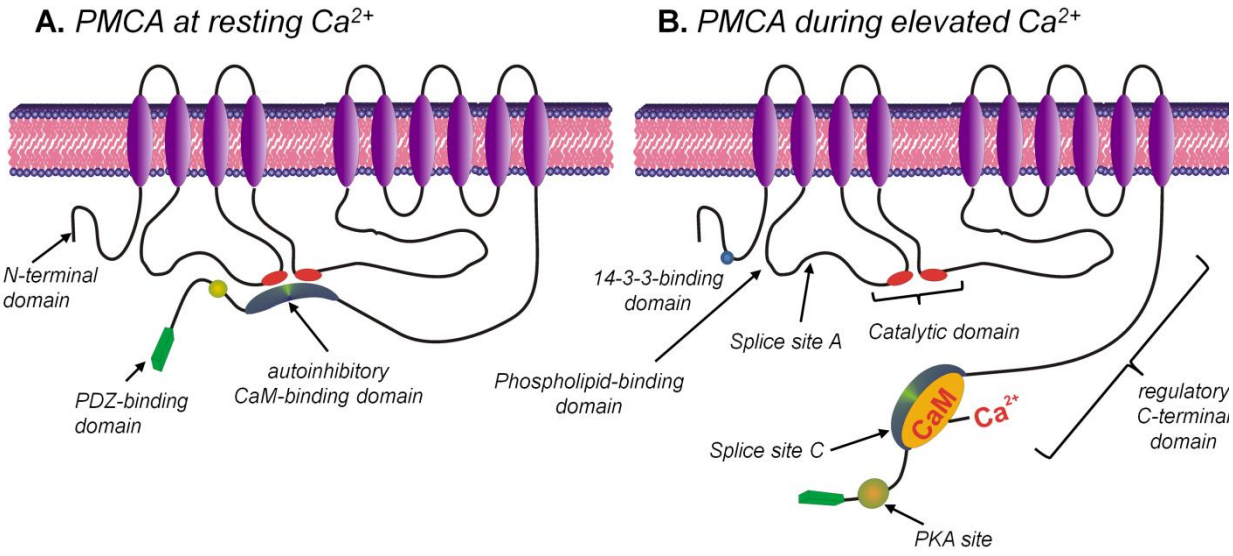

Figure. 1. Cartoon depicting the main structural features of the PMCA at resting $[Ca^{2+}]_i$ (A) and during elevated $[Ca^{2+}]_i$ (B). The PMCA consists of an N-terminal domain, 10 transmembrane domains, 4 cytosolic loops and a regulatory C-terminal domain. When $[Ca^{2+}]_i$ is elevated the Ca/CaM binds to the autoinhibitory CaM-binding domain causing a conformational change which exposes the catalytic site which consists of the ATP-binding sites and the aspartate residue that is phosphorylated during the reaction cycle. This increases the Ca^{2+}-transporting activity of the PMCA.

The first cytosolic loop of the PMCA, which spans between the second and third transmembrane domains, contains numerous key functional domains. These include a stimulatory acidic phospholipid-binding domain (10, 121), part of the binding site for the autoinhibitory calmodulin (CaM)-binding domain (41) (see Figure 1B) and splice site A important for the apical membrane targeting in epithelial cells (28, 48, 57). The second cytosolic loop between the fourth and fifth transmembrane domains contains the major catalytic site. This includes the aspartate residue that becomes phosphorylated during the reaction cycle, the ATP binding domain and the second part of the binding site for the autoinhibitory CaM-binding domain within the C-terminal tail (40). Finally the C-terminal tail contains important regulatory domains of the PMCA, which includes the autoinhibitory CaM-binding domain (59), which at rest interacts with the catalytic domain thereby inhibiting the PMCA (see figure 1B). Binding of Ca^{2+}/CaM to this autoinhibitory domain induces a conformational change which reduces its affinity for the catalytic site thereby increasing the Ca^{2+} transporting activity of the PMCA (22).

The C-terminal tail also contains consensus sites for phosphorylation by PKC (76, 91) and PKA (108), which when phosphorylated increases PMCA activity (44, 78, 105, 122) by increasing CaM binding (50). It has been hypothesised that due to the close proximity of the PKA site to the autoinhibitory CaM-binding domain (60), binding of CaM allows the otherwise cryptic PKA site to be accessible for

PKA-mediated phosphorylation (58, 113). Indeed our own work in salivary acinar cells has shown that the apical PMCA activity is differentially potentiated and phosphorylated in a Ca^{2+}-dependent manner (2, 13, 15). The C-terminus also contains additional high affinity allosteric Ca^{2+} binding sites (58) and acidic phospholipid binding site (79, 80). Binding of acidic phospholipids such as phosphatidylinositol (PI) and phosphatidylserine (PS) increases the Ca^{2+} and ATP affinity of PMCA. Phosphoinositide 4,5-bisphosphate (PIP_2) is also a major activator of PMCA and is thought to account for ~50% of the activity of PMCA at rest (22, 23). Therefore, PIP_2 depletion during G-protein-coupled receptor (GPCR) activation is likely to facilitate the increase in $[Ca^{2+}]_i$ by inhibiting PMCA (22, 23) as well as generating IP_3 and activating IP_3-mediated Ca^{2+} release. The C-terminus also contains a critical splice site, which generates a truncated variant (a-variant) which has a reduced CaM affinity to the full length variant (b-variant) (Figure 1) (38). The functional significance of this is unclear as both splice variants are equally effective at restoring resting $[Ca^{2+}]_i$ (9). The last few amino acid residues of the C-terminus of the PMCA contain PDZ-binding domains, which facilitate PMCA dimerisation (114) which further increases PMCA activity (67). In addition, the PDZ-binding domain also facilitates the recruitment of the actin cytoskeleton (118) and numerous scaffolding proteins and signalling complexes. These include; MAGUK (36), SAP (63), CASK (100), NHERF/EBP50 (35), PISP (47) and Ania3/Homer (104). Such targeting only occurs for the full-length b-variants, suggesting specialised signalling roles for different PMCA isoforms. Specifically, PMCA4b functionally interacts with nNOS (81, 101) and calcineurin (18) thereby regulating their downstream signalling pathways.

ATP dependency of PMCA

Intuitively, one might predict that ATP depletion would inhibit PMCA leading to Ca^{2+} overload and necrotic cell death (4, 26, 32). However, this is likely to be an over simplification because there are numerous factors that can influence the ATP sensitivity of the PMCA and also several factors that often accompany ATP depletion that can also separately influence PMCA activity.

Early studies revealed that PMCA has a catalytic ATP binding site (Km~3 µM) and lower affinity regulatory binding site (Km~145 µM) (96). However, more recent studies suggest a more complex ATP dependency (37). Most healthy cells have a resting ATP concentration in the mM range, which raises the important question of how much ATP has to drop before the PMCA is inhibited? Presumably this will depend on the extent of metabolic stress, whether mitochondria or glycolysis is inhibited, the overall metabolic activity of the cell and whether ATP is being rapidly consumed. Secondly, would inhibition of mitochondrial metabolism alone be enough to deplete ATP levels sufficiently to inhibit the PMCA activity especially if glycolytic ATP production remains active? The classic textbook view is that mitochondria contribute ~95 % of ATP (i.e. 32 molecules of ATP per glucose molecule), whereas glycolysis provides only 5 % (i.e. 2 molecules of ATP per glucose molecule). This is likely to be an over simplification as most cells exhibit metabolic plasticity and are able to adapt to their environment, for example during hypoxia. Cancer cells are an extreme example of this and often undergo a dramatic switch from mitochondrial metabolism to glycolytic metabolism due to mutations of key mitochondrial enzymes and up-regulation of glycolytic enzymes (89, 90). This is known as the Warburg effect, named after Otto Warburg, who first described this in the 1920s (115). Our ongoing recent work on pancreatic cancer cell lines show that glycolytic inhibitors induce cytotoxic Ca^{2+} overload, ATP depletion, inhibition of the PMCA and the consequent necrotic cell death, whereas mitochondrial inhibitors had no effect (unpublished data). This suggests that glycolytically-derived ATP, rather than mitochondrially-derived ATP is critical for maintaining PMCA function and pancreatic cancer cell survival.

Regulation of the PMCA by acidic phospholipids

An important caveat when considering the ATP-sensitivity of the PMCA is that acidic phosphoplipids, such as phosphatidylinositol (PI) and phosphatidylserine (PS) increase the ATP sensitivity of the PMCA and mimic regulation by CaM (79, 80). In particular, the affinity for ATP was as low as 5-10 mM (regulatory site) when PS (or PI) was absent from the lipid environment of the PMCA using cell-free assays (69, 98). This therefore suggests that depletion of PS (or PI) from the membrane may be sufficient to render the PMCA highly sensitive to ATP depletion. However, most of the evidence is based on *in vitro* cell-free assays of ATPase activity, whereby PS/PI was either absent or present in an artificial membrane, which makes it difficult to extrapolate to intact cells. It is therefore unclear what the critical concentration of PS is to maintain "normal" ATP-sensitivity of the PMCA or whether this relationship is influenced by dynamic changes in Ca^{2+}, Mg^{2+}, CaM or lipid environment. However, functional studies in intact endothelial cells have shown that the loss of phosphatidylserine from the inner leaflet of the plasma membrane, following cholesterol depletion with β-methyl-cyclodextran, inhibited PMCA activity (120). This has important implication for apoptosis, since PS is known to line the inner leaflet of the plasma membrane and a proportion is thought to flip to the extracellular side of the membrane during apoptosis (39). This provides the dying cell with an "eat me" signal detected by macrophages that then phagocytose the dying cell from the tissue (39). Furthermore, the enzyme responsible for this PS asymmetry within the plasma membrane (aminophospholipid translocase or flippase) (34), requires millimolar ATP (103, 106) and is inhibited by oxidative stress (16, 56). Collectively these studies suggest that cellular stress may have a profound effect on the ATP sensitivity of the PMCA and thus inhibit the PMCA even with only mild ATP depletion. However, more work is needed to fully understand the complex relationship between membrane phospholipids, ATP and PMCA activity.

Effect of mitochondrially-derived reactive oxygen species

Severe mitochondrial stress, whatever the mechanism, often leads to the generation of reactive oxygen species (ROS) (95). ROS are generated by incomplete reduction of oxygen during the process of oxidative metabolism. In fact, ~1–5% of electrons 'escape' the electron transport chain to generate superoxide ($O_2^{\cdot-}$) (95). The principal source of superoxide is complex III (66) and complex I (19) which in turn can be converted to H_2O_2 by superoxide dismutase and released by mitochondria. There is also good evidence that oxidants (H_2O_2) can directly oxidise PMCA and also oxidise calmodulin, which is the main activator of PMCA (119). Hence, metabolically derived ROS may have a profound inhibitory effect on PMCA activity.

In addition, H_2O_2 has been reported to reduce the functional expression of PMCA at the plasma membrane of cultured hippocampal neurons within 1-2 hours (65). Such rapid changes in functional expression of PMCA at the plasma membrane could lead to reduced Ca^{2+} efflux during metabolic stress even in the presence of continued high ATP levels.

Calpain/caspase cleavage of the PMCA

The release of cytochrome C from the mitochondria and the subsequent activation of caspases and calpain (55) have both been reported to cleave and eventually lead to the inactivation of the PMCA (11, 51, 102). It is interesting to note that the time-frame over which cytochrome C release can occur (> 2 mins) (46) coincides with the time the PMCA can be observed to be inhibited, and well before ATP depletion was observed (3).In fact the initial cleavage by caspase and calpain actually activates the pump, but through the subsequent internalisation and degradation of the PMCA the protease effect is manifested as pump inhibition (27, 92). Specific caspase-3 cleavage of PMCA4b produces a 120 kD fragment that is constitutively activated, due to the removal of the autoinhibitory domain (85-87). It is also interesting to note that calpain can also be directly activated by H_2O_2 and Ca^{2+} (116).

2. PMCA in the pancreas

Compared to the study of other Ca^{2+} transport pathways, such as IP_3 receptors, store-operated Ca^{2+} entry (SOCE) and sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), there has been very little work focussing on PMCA function in pancreatic exocrine cells. Early *in vitro* studies of ATPase activity and Ca^{2+} flux in isolated pancreatic acinar cell membranes revealed that the PMCA has a high affinity for Ca^{2+} (Kd, ~2 μM) and is activated by CaM, PKA, PKC and phospholipids, consistent with studies in red blood cell membranes (1, 62, 72, 73). However, it is difficult to translate these findings to live intact cells due to the dynamic changes in cytosolic Ca^{2+}, ATP, phospholipid composition of the membrane and other signalling molecules. Therefore, studies in intact pancreatic acinar cells have confirmed some of these early findings and importantly have revealed that PMCA was the major, if not only, Ca^{2+} efflux pathway in pancreatic acinar cells due to the lack of functional expression of the Na^+/Ca^{2+}-exchange (NCX) (77). This was later confirmed using the droplet technique; an elegant approach for visualising Ca^{2+} efflux from single cells or clusters of cells, using dextran-conjugated fluorescent dyes trapped within a very small extracellular volume beneath a droplet of oil (110, 111). Using this technique, Ca^{2+} efflux via the PMCA, appeared to be much greater at the apical membrane of pancreatic acinar cells (5-7). Moreover, immunofluorescence studies confirmed that the PMCA is expressed with a much higher density at the apical and lateral membranes of pancreatic acinar cells (68). This apically confined Ca^{2+} efflux occurred during each apically-confined, IP_3R-mediated, cytosolic Ca^{2+} spike during agonist-evoked $[Ca^{2+}]_i$ oscillations (110, 111). This led to the "Ca^{2+} tunnelling" hypothesis, proposed by Ole Petersen's group (75). Since apically confined Ca^{2+} release leads to apical Ca^{2+} efflux, via the PMCA, the ER needs to be replenished to sustain these apically confined $[Ca^{2+}]_i$ oscillations. This was shown to occur by Ca^{2+} entry across the basolateral membrane and the subsequent re-uptake of Ca^{2+} into the ER by basolateral SERCA, thus allowing Ca^{2+} to "tunnel" through to the ER thereby facilitating regenerative apical Ca^{2+} release (75). Furthermore, this apical Ca^{2+} efflux into the pancreatic duct lumen activates Ca^{2+}-sensing receptors (CaSR) which line the apical membrane of pancreatic duct cells thereby facilitating bicarbonate and thus fluid secretion (14). This has been suggested to be an important protective mechanism controlling the lithogenicity of pancreatic fluid and preventing pancreatic stone formation (14).

In the related salivary acinar cells we have also shown that the apical PMCA activity could be differentially potentiated by a Ca^{2+}-dependent, PKA-mediated phosphorylation also important for facilitating maximum fluid secretion (2). Taken together these studies have elevated the importance of the PMCA from a minor house-keeping role to critical roles in the spatiotemporal shaping of $[Ca^{2+}]_i$ signalling and the control of fluid secretion and lithogenecity in the pancreas and salivary glands.

In pancreatic islets all 4 isoforms, and numerous splice variants, of the PMCA have been shown to be expressed, using immunocytochemistry, western blotting and RT-PCR (45, 61, 99, 112). Specifically, PMCA 1 and 4 are ubiquitously expressed in all cells of the islets, whereas PMCA3 and the splice variants PMCA1b, PMCA2b and PMCA4b are reported to be exclusively expressed in β-cells (45, 61, 112). Interestingly it has been shown functionally that elevated glucose has an inhibitory effect on PMCA activity in rat pancreatic islets/β-cells (99, 117). Although the mechanism for this inhibition remains obscure it has been suggested that there is a switch from PMCA function as the major Ca^{2+} efflux mechanism (at low $[Ca^{2+}]_i$) to NCX function (at high $[Ca^{2+}]_i$). It is also important to note that many of these studies use cell free assays of ATPase activity as a measure of PMCA, which makes it difficult to extrapolate PMCA function to intact live cells.

PMCA and cell death

Although the physiological role of the PMCA has been debated for several years, the role of the PMCA during cellular stress and under

pathological conditions is undeniable. The nature of cell death (i.e. necrosis vs apoptosis) will largely depend on the extent of metabolic stress and cytosolic Ca^{2+} overload. However, ATP depletion during extensive metabolic stress has been suggested to be the switch from apoptosis to necrosis (31, 64, 70), regardless of whether this is accompanied by Ca^{2+} overload. This is largely because ATP is required for many of the apoptotic processes, but not for necrosis (70). During pancreatitis, apoptosis is generally regarded as protective as this involves the safe dismantling of the cell constituents (8, 52). Necrosis, on the other hand, is the uncontrolled cell death due to cell lysis and the release of activated proteases (zymogens) which trigger the spiral of self-perpetuating tissue damage characteristic of acute pancreatitis (8, 52). This includes local inflammation and the recruitment of activated neutrophils to the site of cellular injury. This often leads to systemic inflammation especially if the activated zymogens leak into the blood stream causing distal organ damage and thus multiple organ failure (84). Cytosolic Ca^{2+} overload, metabolic stress and necrosis are linked in various ways, but perhaps most critically via the PMCA.

In addition, in the context of cell death and cytoprotection, the impairment of PMCA function and subsequent dysregulation of cytosolic Ca^{2+} homeostasis can, in some cases, be cyto-protective (83). During oxidative stress or tumour necrosis factor (TNF)-induced cell death, the accumulation and damage of lysosomes has been suggested to be important (17, 82). In TNF-resistant cell lines, in which PMCA4 is mutated, the resulting enhanced Ca^{2+} signalling has been shown to promote the exocytotic loss of lysosomes resulting in protection against TNF-induced cell death (83). This therefore suggests, somewhat counter-intuitively, that PMCA4 promotes TNF-induced cell death.

Interestingly, the anti-apoptotic factor Bcl-2/xL has been recently shown to inhibit PMCA activity in pancreatic acinar cells (42). Although the pathophysiological relevance of this phenomena remains to be determined this observation highlights the critical importance of the PMCA in controlling cell fate.

Effect of ATP depletion on PMCA activity during metabolic stress

This will depend on the “extent” of metabolic stress, whether mitochondria or glycolysis is inhibited and how quickly ATP is consumed. We have previously reported that oxidative stress, induced by H_2O_2, impaired hormone evoked Ca^{2+} oscillations, induced an irreversible Ca^{2+} overload and marked inhibition of PMCA in pancreatic acinar cells (3, 12). This H_2O_2-induced PMCA inhibition could occur without mitochondrial Ca^{2+} handling, coincided with mitochondrial depolarization and was sensitive to inhibitors of the mitochondrial permeability transition pore (mPTP) (3). These data were consistent with studies showing that severe ATP depletion can cause inhibition of the PMCA (4, 26, 32). PMCA activity was shown to decrease ~5 fold following the combined treatment with oligomycin and iodoacetate in pancreatic acinar cells (4). Moreover, the pancreatitis-inducing agents, palmitoleic acid ethylester (POAEE), palmitoleic acid (POA) and bile acids have all been shown to induce Ca^{2+} overload, mitochondrial depolarisation and ATP depletion (32). This POAEE-induced Ca^{2+} overload, which was due to ATP-depletion-induced inhibition of the PMCA and SERCA, was largely abrogated by replenishment of ATP via a patch pipette (32). However, our recent studies have shown that the H_2O_2-induced Ca^{2+} overload, ATP depletion and inhibition of the PMCA were abrogated by pre-treatment with insulin (74), which has recently been extended to POA-induced cytotoxicity (unpublished data). This insulin protection was independent of oxidative stress and mitochondrial depolarisation but involved activation of PI3K/Akt and a switch from mitochondrial metabolism to glycolytic metabolism which was sufficient to preserve the ATP supply to the PMCA, thereby preventing cytotoxic Ca^{2+} overload (74).

Incidentally the PMCA has been shown to have its own localised glycolytic ATP supply, which under certain conditions may render it largely insensitive to inhibition of mitochondrial metabolism (53, 54, 88). Specifically, studies in isolated inside-out plasma membrane vesicles from pig stomach smooth muscle enriched with

PMCA showed that an endogenous membrane-bound glycolytic system provided ATP to fuel the PMCA-dependent Ca^{2+} uptake (53, 54). As long as glycolytic substrates were present the Ca^{2+} uptake (PMCA activity) persisted in the absence of an exogenously applied ATP regenerating system (53, 54). Furthermore, studies in red blood cells have shown that several glycolytic enzymes associate with the plasma membrane, either via band 3 protein (anion-exchanger) (20, 21, 29, 94) or via phospholipids (33). Moreover, the PMCA has been shown to reside within caveolae, where these phospholipids are enriched and regulate the activity of the PMCA (120). Finally, it has been suggested that a localised pool of ATP, associated with the cytoskeleton, provides a privileged ATP supply to the PMCA (30). Collectively these data have important implications for the ATP-regulation of the PMCA.

Summary

The PMCA is critical for maintaining low resting cytosolic Ca^{2+} and for preventing cytotoxic Ca^{2+} overload and cell death, particularly in cells in which the NCX is either not expressed or has minimal functional activity, such as pancreatic acinar cells. In addition, there is increasing evidence that the PMCA may physically and functionally couple to a variety of signalling pathways and metabolic enzymes, elevating its importance as a general house-keeping role to the control of dynamic cell signalling in both the exocrine and endocrine pancreas. Since the PMCA is an ATP-driven pump, severe metabolic stress and ATP depletion during pathological situations should lead to inhibition of the PMCA and thus Ca^{2+} overload and cell death. Although this relationship is likely an over-simplification the importance of the PMCA in diseases such as pancreatitis, pancreatic cancer and diabetes warrant further investigation.

3. Tools available to study the PMCA

a. cDNA clones

A variety of PMCA containing plasmids can be obtained from Emanuel Strehler (Mayo Clinic, Rochester, MN). These include DNA constructs encoding EGFP fused to PMCA2b or PMCA4b (36) and DNA constructs encoding the truncated PMCA that lacks the PDZ-binding domain (EGFP-PMCA2b□6 & EGFP-PMCA4b□6) (28).

b. Antibodies

Non isoform-specific PMCA ATPase Antibody (clone 5F10) (Mouse monoclonal; MA3-914; Thermo Scientific, Pierce Antibodies)

PMCA1-specific ATPase Antibody (clone 5F10) (rabbit polyclonal; PA1-914; Thermo Scientific, Pierce Antibodies)

PMCA2-specific ATPase Antibody (rabbit polyclonal; PA1-915; Thermo Scientific, Pierce Antibodies)

PMCA3-specific ATPase Antibody (rabbit polyclonal; PA1-916; Thermo Scientific, Pierce Antibodies)

PMCA4-specific ATPase Antibody (clone JA9) (Mouse monoclonal; MA1-914; Thermo Scientific, Pierce Antibodies). JA9 reacts specifically with a region containing residues 51–75 of hPMCA4 (a or b), but not with the same region of PMCA1, 2 or 3 (24).

Santa Cruz Biotech has produced a range of PMCA4b-specific mouse monoclonal, including clone JA3, 2T2 and 3F18. JA3 reacted with residues 1156–1180, a region unique to hPMCA4b (24).

Other splice variant specific polyclonal antibodies that recognise the a-splice variants of PMCA1, 2, 3 and 4 directed against the C-terminal domains of each PMCA isoform termed CR1a, CR2a, CR3a and CR4a and thus recognise PMCA1a, 2a, 3a and 4a respectively have been described (43).

c. Mouse Models

A range of PMCA knockout mice have been created by numerous groups but most noticeably by Gary Schull (University of Cincinnati College of Medicine, Cincinnati, Ohio) (93) and Lugwig Neyses (University of Manchester, UK). The CByJ.A-*Atp2b2*$^{dfw\text{-}2J}$/J mouse strain which represents the PMCA2

knockout can be obtained from Jackson Labs (http://jaxmice.jax.org/strain/002894.html)

d. In situ Ca^{2+} clearance Assay

We have developed an *in situ* $[Ca^{2+}]_i$ clearance assay in which all other Ca^{2+} transport is inhibited in live intact cells. While *in vitro* cell-free assays of ATPase activity and $^{45}Ca^{2+}$ flux measurements have provided valuable information in characterising the PMCA function, these techniques are severely limited. This is because PMCA activity in a live cell can be influenced by dynamic changes in Ca^{2+}, ATP, numerous cytosolic factors and the phospholipid environment within the plasma membrane. Further details of our *in situ* $[Ca^{2+}]_i$ clearance assay can be found in reference 2 and 68 and figure 2 below.

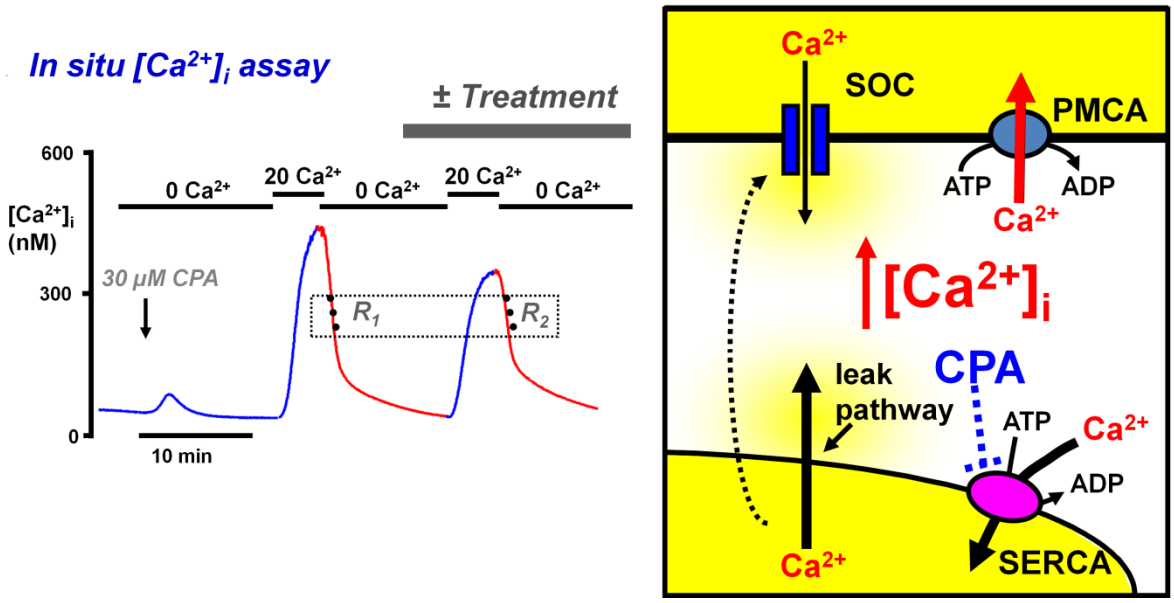

Figure 2. *In situ* $[Ca^{2+}]_i$ clearance assay of PMCA activity in live intact pancreatic acinar cells. This involves treating cells with 30 μM cyclopiazonic acid (CPA) in zero external Ca^{2+} (1 mM EGTA), which inhibits SERCA, thereby depleting the ER of Ca^{2+}. This lead to a small increase in $[Ca^{2+}]_i$ which recovered to baseline presumably due to the PMCA. The addition of 20 mM Ca^{2+} leads to a large increase in $[Ca^{2+}]_i$ due to store-operated Ca^{2+} entry which then reaches a short-lived steady state. Subsequent removal of external Ca^{2+} (1 mM EGTA) leads to clearance of $[Ca^{2+}]_i$ due predominantly to the PMCA. Mitochondrial Ca^{2+} uptake may contribute to this net $[Ca^{2+}]_i$ clearance but this can be abolished using the mitochondrial Ca^{2+} uptake inhibitor, Ru360 (see Ref 2). This influx-clearance phase can be repeated in the presence of a test reagent/treatment using a paired experimental design and the rate normalised to the initial clearance of the same cell. This normalised rate can then be compared to time-matched control experiments (see Ref 3 and 74).

e. Characterisation of PMCA activity in pancreatic acinar cells

We have shown that following treatment with the SERCA inhibitor, cyclopiazonic acid (CPA), $[Ca^{2+}]_i$ clearance was inhibited using the PMCA-specific inhibitor, carboxyeosin diacetate (figure 3b). Under conditions of the *in situ* $[Ca^{2+}]_i$ clearance assay the NCX has no role, as $[Ca^{2+}]_i$ clearance was unaffected by replacing all external Na^{2+} with N-methyl-D-glucamine (NMDG) (figure 3D). Further details can be found in reference 2 and 68 and figure 3 below.

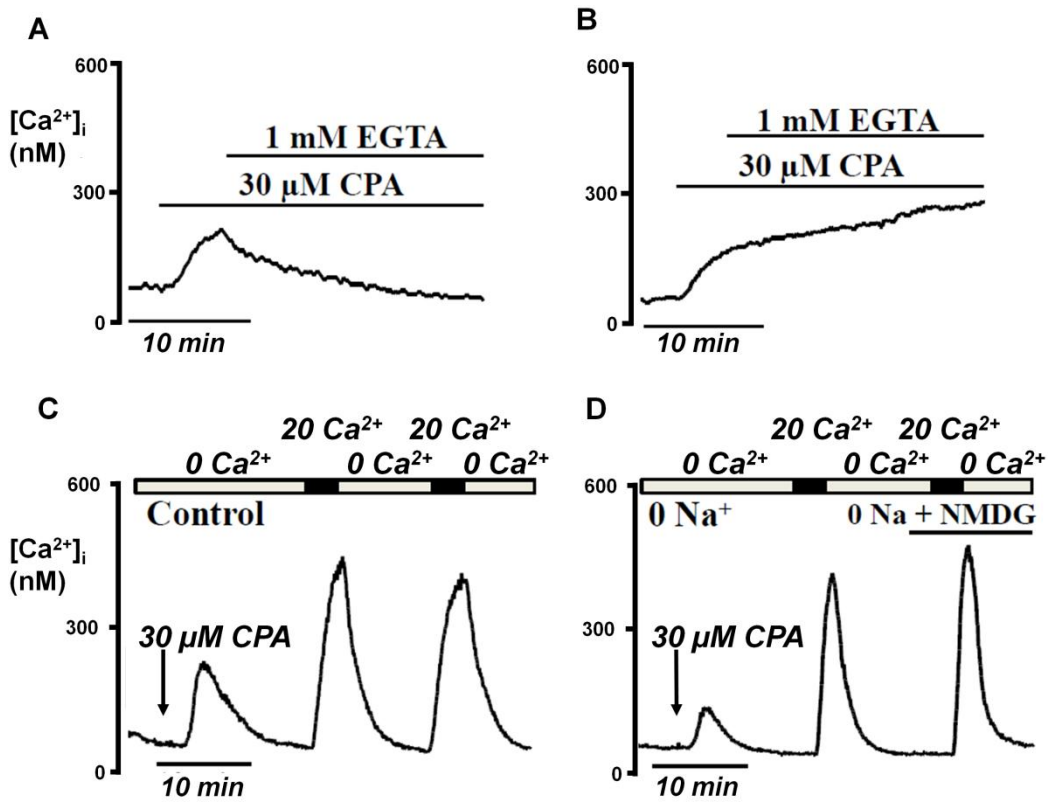

Figure 3. Characterisation of PMCA activity in pancreatic acinar cells. Cell were pre-incubated without (***A***) or with (***B***) carboxyeosin diacetate to inhibit the PMCA. Treatment with CPA caused a leak of Ca^{2+} from the ER and subsequent activation of SOCE. Subsequent removal of external Ca^{2+} (1 mM EGTA) caused $[Ca^{2+}]_i$ to be rapidly cleared from the cytosol of control cells (***A***) but not from carboxyeosin-treated cells (***B***). Using the *in situ* $[Ca^{2+}]_i$ clearanceassay, replacing all external Na^+ with N-methyl-D-glucamine (NMDG) had no effect on the rate of clearance, suggesting that the NCX has no role.

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