Deletions and Mutations in the Acidic Lipid-binding Region of the Plasma membrane Ca\(^{2+}\) pump: a study on different splicing variants of isoform 2

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Running title: functional analysis of PMCA2 splicing variants

Acidic phospholipids increase the affinity of the PMCA pump for Ca\(^{2+}\). They interact with the C-terminal region of the pump and with a domain in the loop connecting transmembrane domains 2 and 3 (A\(_L\) region), next to site A of alternative splicing. The contribution of the two phospholipid binding sites and the possible interference of splicing inserts at site A with the regulation of the ATPase activity of isoform 2 of the pump by phospholipids have been analyzed in microsomal membranes of CHO cells overexpressing the full-length z/b variant (no insert at site A), and the w/a variant, containing both the 45 amino acids A-site insert and a C-site insert that truncates the pump in the calmodulin binding domain. The A-site insertion did not modify the phospholipid sensitivity of the pump, but the doubly-inserted w/a variant became insensitive to acidic phospholipids, even if containing the intact A\(_L\) phospholipid binding domain.

Pump mutants in which 12-amino acids had been deleted, or single lysine mutations introduced, in the A\(_L\) region were studied by monitoring agonist-induced Ca\(^{2+}\)-transients in overexpressing CHO cells. The 12 residue deletion completely abolished the ATPase activity of the w/a variant but only reduced that of the z/b variant, which was also affected by the single lysine substitutions in the same domain. A structural interpretation of the interplay of the pump with phospholipids, and of the mechanism of their activation, is proposed on the basis of molecular modeling studies.

The plasma membrane Ca\(^{2+}\) ATPases (PMCA) extrude Ca\(^{2+}\) from cells, maintaining the resting level of intracellular Ca\(^{2+}\) and controlling the Ca\(^{2+}\) transients induced by agonists. Four basic PMCA isoforms are encoded by four independent genes. PMCA1 and 4 are ubiquitously expressed, whereas PMCA2 and PMCA3 are restricted to brain, muscles, and few other tissues: the tissue-restricted isoforms are more active in exporting Ca\(^{2+}\) than the ubiquitous isoforms (1), probably due to their higher affinity for the activator calmodulin. The transcript of each gene is subjected to alternative splicing at sites A and C. About 30 splice variants have so far been detected at the RNA or protein levels (2).

The architecture of the PMCA predicts 10 transmembrane domains, two large intracellular loops, and N- and C-terminal cytoplasmic tails. The 90 residues N-terminal portion appears not to have specific functions even if it contains a consensus binding site for the 14-3-3 protein, which inhibits three of the four pump isoforms (3,4). The cytosolic loop between transmembrane domains 2 and 3 contains a site that binds activatory acidic phospholipids and site A of alternative splicing upstream of it. Pump variants containing the A-splice-site-insert are targeted to the apical plasma membrane (5), and the insert has recently been suggested to have a role in the interactions of the pump with lipids in the plasma membrane (6). The C-terminal tail contains other regulatory sites of the pump, among them the positively charged calmodulin binding domain, which also binds acidic phospholipids (7), the consensus sites for protein kinases A (PKA, isoform-specific) and C (PKC), and high affinity allosteric Ca\(^{2+}\) binding sites.
Under non-activated conditions, the C-terminal tail of the pump is proposed to fold over to interact with two sites in the first and second cytosolic loops of the enzyme, compromising the access to the active centre. Calmodulin then interacts with its binding domain, removing it from its docking sites next to the active centre, and freeing the pump from autoinhibition. The calmodulin regulation of the pump has been extensively investigated and is now well understood, but that mediated by acidic phospholipids is still unclear. Acidic phospholipids enhance the Ca\textsuperscript{2+} sensitivity of the pump. The order of stimulatory potency [PtdIns(4,5)P\textsubscript{2} > PtdIns4P > PtdIns ~ phosphatidylserine (PS) ~ phosphatidic acid (PA)] is proportional to the number of negative charges on the lipids (12). The stimulation is appreciably reduced by complexing the negative charges with polyamines or neomycin (13). Recently, diacylglycerol (DAG) has also shown to be a stimulator of the PMCA. Interestingly, the activation induced by DAG is additive to that produced by calmodulin and protein-kinase C, suggesting that DAG interacts with the PMCA through a specific mechanism (14).

The acidic phospholipid binding region next to splice A was recently deleted in a variant of PMCA4 containing an inserted exon at splicing site A (variant \textit{xb}) (15,16). Partial deletions did not alter Ca\textsuperscript{2+} transport activity, but made the pump insensitive to acidic phospholipids. However, complete removal of the domain made the pump inactive (15).

The contributions of the two phospholipid binding sites, and of the alternative splicing at site A next to one of them, to the regulation of the pump have not been analyzed. It was felt interesting to study these aspects on isoform 2 of the pump, as this isoform has very high activity even in the absence of calmodulin (17,18), but responds to acidic phospholipids in the same way of PMCA4 (17). In addition, PMCA2’s splicing mechanisms generate a larger number of variants than in other isoforms: up to three exons are inserted at site A, generating variant \textit{z} (no exons included), variant \textit{y} (two exons included), variant \textit{x} (one exon included) and variant \textit{w} (all three exons included). Splicing at site C excludes 2 novel exons (variant \textit{b}, full length) or includes them (variant \textit{a}). The \textit{a} insertion leads to a truncated version of the pump that only contains about half of the original calmodulin binding domain (17,18). We had previously reported that the \textit{z/a} and \textit{w/b} PMCA2 variants behaved essentially as the full-length, non-inserted, \textit{(z/b)} pump (perhaps, they were slightly less efficient) (1,19,20). The doubly inserted \textit{w/a} PMCA2 variant had only limited ability to rapidly increase activity when challenged with a Ca\textsuperscript{2+} pulse, but had about the same high non-stimulated (basal) activity of the full-length \textit{z/b} variant (19).

This contribution explores the activation of splicing variants of isoform 2 of the PMCA pump by acidic phospholipids. Since the negative charges on the lipids are likely to be important in the stimulatory effect, the study was performed using a pump variant in which a 12 residue stretch in the \textit{AL} acidic phospholipids binding domain, which contains 4 positively charged residues, was removed. Point mutations that selectively substituted positive residues (K), or two other conserved polar residues (S and E) were also introduced in the stretch. The scheme of Figure 1 summarizes graphically the details of the PMCA2 variants and mutants used in this study.

**Experimental Procedures**

**Cell cultures and transfection**

CHO cells were cultured in F-12 Ham nutrient mixture (Invitrogen), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (60 µg/µl) and streptomycin (120 µg/µl) in 75cm\textsuperscript{2} Falcon flasks at 37°C. For the microsomes preparation, CHO cells were plated on 150x25 mm Petri dishes allowed to grow to 50% confluence and transfected according a calcium–phosphate procedure with 30 µg of total plasmid DNA. For the aequorin and immunocytochemistry experiments, CHO cells were plated onto 13 mm glass coverslips, allowed to grow to 50% confluence and transfected according a calcium–phosphate procedure with 3 µg of total plasmid DNA or with 1.5 µg of each plasmid DNA in the case of co-transfection. GFP-tagged PMCA2 \textit{z/b} and \textit{w/b} are of human origin and GFP-tagged PMCA2 \textit{w/a} variants (wt and del12 mutant) are from rat. Untagged PMCA pump variants (\textit{w/a} and \textit{z/b}) of human origin were also used in the Ca\textsuperscript{2+} measurements experiments in living cells. No differences were observed between the GFP-tagged and untagged PMCA2 activity.

The average transfection efficiency approached 25% and the increase of PMCA protein in overexpressing cells, calculated by densiometric analysis of Western blotting showing the endogenous PMCA (i.e., blots developed with the monoclonal antibody 5F10 which recognized all PMCA isoforms) and corrected for the whole cell population, would correspond to about 3 fold the endogenous level (data not shown).
Microsomal membranes preparation from CHO cells

Cells from five 150x25 mm dishes were washed once with Phosphate Buffer Saline (PBS) containing 1 mM EDTA and harvested in 10 ml of PBS containing 0.1mM phenylmethylsulfonyl fluoride (PMSF) and Cocktail proteases inhibitors EDTA-free (Roche). Cells were collected by centrifugation (2000 g, 10 min) at 4°C and resuspended in 6 ml of a hypotonic solution of 10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 0.1 mM EDTA, Cocktail proteases inhibitors EDTA-free, and 2 mM dithiothreitol (DTT). The cells were swollen for 15 min on ice and then subjected to three cycles of freeze and thaw. The homogenate was diluted with an equal volume of 0.5 M sucrose, 0.3 M KCl, 2mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, homogenized again with three cycles of freeze and thaw, and centrifuged at 5000 g for 15 min. KCl was added up to 0.6 M in the supernantant and, in order to remove calmodulin, an excess of EDTA (1.5 mM) was also added. The suspension was centrifuged at 100.000 g for 40 min to pellet the microsomal fraction. The final pellet was resuspended in a solution containing 0.25 M sucrose, 0.15 M KCl, 10 mM Tris-HCl, pH 7.5, 2 mM DTT, and 20μM CaCl2, at a protein concentration of 1-3mg/ml and stored in liquid N2.

ATPase activity assay

The ATPase activity was measured by the coupled enzyme assay (modified from (21)) monitoring the adsorbance of NADH at 340 nm. The decrease in OD340 can be converted into ATPase activity by a factor considering the NADH molar extinction coefficient (εNADH) and the amount of protein (μg). The real activity was obtained subtracting the basal activity from the maximal activity. The assay was performed at 37°C in a final volume of 1 ml of a mixture containing 20 mM Tris-HCl, pH 7.2, 5 mM MgCl2, 0.5 mM EGTA, 0.1 M KCl, 0.5 mM phosphoenolpyruvate (PEP), 0.15 mM NADH, 1.4 U piruvate kinase/lactic dehydrogenase (PK/LDH, Roche), 4 mM ATP, 25 μg PMCA membranes and 50 μM CaCl2. The ATPase activity, detected at 340 nm (DU640 Spectrophotometer, Beckman Coulter) was expressed in μmol P_i/min/mg of protein (Phosphate moles originated from the ATP hydrolysis): the maximal activity and the basal activity were calculated by multiplication of the activity curve slope value by a factor considering the NADH molar extinction coefficient (εNADH) and the amount of protein (μg). The real activity was obtained subtracting the basal activity from the maximal activity. The assay was performed in the presence of 5μg/ml olygomicin and 0.1 μM thapsigargin. To test calmodulin (CaM) or phosphatidylerine (PS) activation of the pump 200 nM CaM or 25 μM PS were preincubated with the membranes for 5 minutes at 37°C before starting the assay. Generation of PMCA2z/b del12 and PMCA2z/b mutants expression plasmids.

To generate PMCA2z/b with the deletion of 12 amino acids in the domain that bind acidic phospholipids, two PCR amplification products that did not contain the portion of 12 amino acids were generated using 4 different primers bearing restriction sites for EcoRI/HindIII and HindIII/BamHI: 5’cggGAATTCatggctgcatgaccaac3’; 5’cggTTCGAAgtcgcagtgccttc3’; 5’cggTTCGAAgtcgcagtgcggaag3’; cggGATCCtaaagcgacgtcgccag. The PCR products were digested with the respective restriction enzymes and were inserted in a three part ligation reaction in pcDNA3 vector (Invitrogen) digested with EcoRI and BamHI. The construct was controlled by sequencing. In vitro site-mutagenesis in the PMCA2z/b was carried out with QuikChange II Site-Directed MutagenesisKit (Stratagene) according to the manufacturer’s instructions using the following primers:

K336 sense 5’ ccagcatgcaagcGGgagaaatgcgcgc 3’
K336 antisense 5’ gcagccatctctcGCcctggtcgtgcgg 3’
K338 sense 5’ tcgacaagaagggCGtgcggtgcgtgagcgg 3’
K338 antisense 5’ ccgctgcaagggGCctcctctgtgcagg 3’
K344 sense 5’ cgtgctgcagggGCgtcgaacacgcgtgagcgg 3’
K344 antisense 5’ ccgcttggaggcGCgcggccagcagcgg 3’
K347 sense 5’ ggggacagctcgcGgtggtgcgtgcagatcgg 3’
K347 antisense 5’ atctgcagacgcAGGgtggtgcgtgccc 3’
E337 sense 5’ gacatcagcaagaggGCgatgcggtggccgcgcgg 3’
E337 antisense 5’ gctcgtcagcagggCTctgcctgatgcgg 3’
S339 sense 5’ gacatcagcaagggGcaacacggtggtgcgtgagcgg 3’
S339 antisense 5’ gcggctgcaagggGCgcttcctctgatgcgctgag 3’

The PMCA2_4M mutant, in which all the four lysines were mutated, was generated by subsequent cycles of PCR amplification using the following primers:

K336_338 sense 5’ ccagcatgcaagaGCGggagGCGtgcggtgagcgg 3’
CHO cells were plated on 13 mm glass coverslips and transfected according to the calcium-phosphate procedure. 36 h after transfection, the cells were incubated for 3 hours with 5 μM of the aequorin prosthetic group coelenterazine wt (wild type) in Dulbecco's modified Eagle's medium supplemented with 1% FBS at 37°C in a 5% CO2 atmosphere.

After incubation with coelenterazine the coverslips were placed in a perfused thermostatted (37°C) chamber of a luminometer positioned in close proximity to a low noise photomultiplier, with a built-in amplifier discriminator. The experiments were performed in a Krebs-Ringer medium (135 mM NaCl, 5 mM KCl, 0.4 mM KH2PO4, 1 mM MgSO4, 20 mM Hepes, pH 7.4 at 37°C) (KRB) supplemented with 0.1% glucose and 1mM CaCl2. The cytoplasmic Ca2+ concentrations were measured after addition of 100 μM InsP3-generating agonists ATP. The experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca2+-rich solution (10 mM CaCl2 in H2O) to discharge the remaining aequorin pool. The light signal from the discriminator was collected by a Thorn-EMI photon counting board and stored in an IBM-compatible computer for further analysis. The aequorin luminescence data were calibrated offline into [Ca2+] values, using a computer algorithm based on the Ca2+ response curve of wild type aequorin (22).

In silico analysis

The protein sequence of human PMCA2 was retrieved from the NCBI database (accession number NP_001674) (23) and amino acid conservation was evaluated with Conseq (24). Secondary structure and disorder were predicted respectively by a consensus approach (25) and SPRITZ (26). A consensus of three methods (Prodiv-TMHMM, HMMTOP and PHOBIUS) was adopted to predict the transmembrane regions. A homology-derived 3D structure model of Human PMCA2 was constructed using the Homer-A modelling server (URL: http://protein.bio.unipd.it/) based on the PDB structure 2agv (chain A) of sarco/endoplasmatic Ca2+-ATPase (SERCA1a). The loop insertions in human PMCA2 were modelled using a divide and conquer method (27). The C-terminal PMCA2 CaM binding region in complex with calmodulin was modelled using the PDB structure 2KNE as template. We used the Pymol Molecular Graphics System (DeLano Scientific, San Carlo, CA, USA, http://www.pymol.org/) to map the residue positions in the protein structure and visualize the
electrostatic surface calculated by the APBS tool (Adaptive Poisson-Bolzman Solver) (28).

**Statistical analysis**

Data are reported as means ± sd. Statistical differences were evaluated by Student’s 2-tailed t-test for unpaired samples, with p value 0.01 being considered statistically significant.

**Results**

**Expression of PMCA2 isoforms in CHO cells**

GFP-tagged PMCA2 splice variants z/b, w/b, and w/a were overexpressed in CHO cells. Crude membranes were prepared and 20 μg of total proteins were separated by SDS PAGE and blotted onto nitrocellulose filters. The filter was incubated with polyclonal antibody 2N that recognizes the PMCA2 isoform, and with an antitubulin antibody. Figure 2 shows that all three splice isoforms of the pump were expressed at approximately equivalent levels.

**Ca\(^{2+}\) ATPase activity in microsomal membranes**

Microsomal membranes (containing plasma membrane fragments/vesicles) isolated from transfected cells were assayed in the presence of thapsigargin and oligomycin to inhibit the activity of the endogenous SERCA pump, and the ATP linked Ca\(^{2+}\) uptake by mitochondrial vesicles that could possibly contaminate the microsomal preparation. Figures 3A and 3D show the PMCA activity in the absence of calmodulin. Both the non-inserted full length PMCA2 z/b variant and the w/b variant had higher basal activity than the inserted and truncated isoform w/a. The calmodulin sensitivity of each isoform was investigated at a fixed Ca\(^{2+}\) concentration in the presence of excess (200 nM) calmodulin (Figures 3B and 3D). As already shown by previous work, the w/a variant had reduced stimulation by calmodulin than the full length z/b and w/b variants.

The splicing event at site A occurs just upstream of one of the two regions responsible for the binding of acidic phospholipids. The first two spliced exons of PMCA2 encode a relatively hydrophobic stretch of amino acids positioned amidst a highly charged region, suggesting possible effects on the overall interaction of the first cytosolic loop of the pump with acidic phospholipids. The response of the three pump variants z/b, w/b and w/a to phosphatidylserine was thus compared. Figures 3C and 3D show that isoforms z/b and w/b had the same response, implying that the A\(_L\) acidic phospholipid binding domain was not affected by the site A-insert.

Surprisingly, however, the w/a variant, which has the phospholipid binding domain contiguous to the site A insert, but lacks about half of the C-terminal phospholipid binding domain was completely insensitive to phosphatidylserine: the response of the full length variants of the pump (variants b) was over 5 fold higher than that of the truncated w/a variant. The finding thus suggests a predominant role of the C-terminal phospholipid binding domain in the response to acidic phospholipids.

**Mutations in the N-terminal (A\(_L\)) phospholipid binding domain**

Mutational experiments on the phospholipid binding domains were performed to further explore the molecular mechanism of the activation of the pump by acidic phospholipids. As for the possible mechanism of acidic phospholipids stimulation, in the case of the binding sequence in the C-terminal calmodulin binding domain it was reasoned that the head groups of positively charged residues could be neutralized by acidic phospholipids, weakening the autoinhibitory intramolecular interaction of the C-terminal tail of the pump with its receptor sites in the main body of the molecule. The study of the phospholipid binding domain in the C-terminal region was limited to comparison of the a variant (in which the splicing truncation removes about half of the CaM binding domain and, presumably, affects the binding of acidic phospholipid binding domain) with the full length b variant. No mutations were introduced in the full-length C-terminal region of the b variants. In the A\(_L\) region, structural rearrangements of the transduction (activator) and catalytic domains of the pump could occur following the binding of phospholipids that would facilitate the access of Ca\(^{2+}\) to its single high affinity site in the transmembrane sector. The four lysine in the A\(_L\) phospholipid binding region, which are very conserved among the PMCA isoforms (Figure 4A), and in the PMCA across species (Figure 4B), could form a charged bend which could easily accommodate a charged phospholipid head. It was thus decided to mutate them. It was also felt that two other well conserved residues in the A\(_L\) binding domain (E337 and S339, Figure 4) could also have a role in the interaction (see below). It was thus decided to mutate them as well. It was also decided to study the effect of the deletion of the 12 residue lysine-rich stretch in the A\(_L\) domain that had been previously performed by others (29).
Generation, expression and activity of PMCA2zb_del12 and PMCA2wa_del12 mutants

The 12 residues lysine-rich stretch located in the N-terminal portion of the domain was analyzed first, as it had already been shown that the deletion of this stretch failed to affect the plasma membrane targeting of the pump (29). The effect of the deletion of region 380-391 in the w/a variant, which has lost at least half of the C-terminal acidic phospholipid binding domain, and of region 336-347 in the full length z/b variant, which contains it, was studied in expressing CHO cells. The activity of the deleted variants of the expressed pump was compared to that of their respective wild type variants. Appropriate controls (Western blotting and immunocytochemistry analysis) established that the mutant pump variants were expressed at about the same levels with respect to their wt versions, and were correctly delivered to the plasma membrane (Figure 5A). The PMCA2wa_del12 was expressed as a GFP-fusion chimera: the fusion with GFP did not alter the targeting nor the activity of the pump (29). As previously reported (19), the w/a variant was much less efficient than the z/b variant in re-establishing resting cytosolic Ca2+ concentration following the increase induced by the stimulation of the cells with the purinergic agonist ATP, (traces of Panels B and C in Figure 5). The Ca2+ transient generated by the stimulation reflects the InsP3-mediated Ca2+ release from the intracellular stores, but also the Ca2+ influx from the extracellular medium through channels activated by the depletion of the endoplasmic reticulum stores. The lowering of the Ca2+ peak with respect to untransfected cells reflects the ability of the overexpressed pumps to respond with a burst of activation, i.e., of Ca2+ extrusion, to the arrival of the InsP3-generated Ca2+ pulse. The faster clearance of the Ca2+ signal is thus due to increased overall pump activity. Figure 5B shows the Ca2+ response in cells transiently transfected with the wa_wt and with wa_del12 variants of PMCA2. Surprisingly, the deletion of the 12 amino acids in the phospholipid binding domain completely abolished the activity of the pump (the heights of the transients were wa_wt: 2.77 ± 0.35 μM, n = 27; wa_del12: 3.58 ± 0.31 μM, n = 26; control (only aequorin): 3.53 ± 0.48 μM, n= 34). The half time of the declining phase was 7.69 ± 1.23 sec, n= 29 in wa_wt, 44.52 ± 4.99 sec in wa_del12, n= 27 and 46.67 ± 7.35 sec, n= 12 in the control (see inset). Figure 5C shows pump activity in cells transiently transfected with the wt_zb and zb_del12 PMCA2 variants. The 12 amino acid deletion impaired the activity of the zb variant as well (zb_wt: 1.31 ± 0.17 μM, n = 31; zb_del12: 2.72 ± 0.28 μM, n = 10), suggesting that the deleted residues are important to pump activity independently of splicing processes. However, at variance with the wa_del12 variant, the zb_del12 variant was still partially active: the height of Ca2+ transient was reduced with respect to control cells. This finding is also supported by the analysis of the declining phase of the Ca2+ traces, in which the half time of the peak decay was 6.31 ± 0.85 sec, n= 13 in zb_wt, 38.64 ± 5.93 sec in zb_del12, n= 14 vs 46.67 ± 7.35 sec, n= 12 in the control, p<0.01 (see inset).

Generation, expression and activity of PMCA2 z/b variants harboring single K mutations in the 336-347 domain.

Single amino acids mutants of the PMCA2 z/b pump were generated by replacing individual lysines in the 12 residue sequence (336-347 domain). Five mutants were generated: PMCA2zb K336A, PMCA2zb K338A, PMCA2zb K344A, PMCA2zb K347A, and one in which all four lysines were replaced with alanines: PMCA2zb_4M. The positions of the mutated Ks in the sequence are shown in Figure 4. The level of expression of all mutants and their correct delivery to the plasma membrane were checked and found to be equivalent (Figure 6A). The single mutation of three of the four lysines impaired the activity of the pump (the heights of the peak transients induced by the stimulation were 1.50 ± 0.15 μM, n=12 for PMCA2 zb_K338A, 1.96 ± 0.09 μM, n=15 for PMCA2 zb_K344A, and 1.56 ± 0.22 μM, n=15 for PMCA2 zb_K347, vs. 1.31 ± 0.17 μM, n = 31 for the zb_wt, p< 0.01) (Figure 6B, in which the Ca2+ transients were superimposed to that generated in cells overexpressing equivalent levels of PMCA2zb_wt). Figure 6B shows that instead the mutation of lysine 336 (K336A) had no effect on the Ca2+ extruding ability of the pump: the height of the transient was 1.36 ± 0.15 μM, n= 12 as compared to 1.31 ± 0.17 μM, n= 31 in zb_wt expressing cells. The mutation of all 4 lysines impaired the Ca2+ extrusion activity of the pump. The peak height was 1.78 ± 0.16 μM, n= 15 for PMCA2 zb_4M, vs. 1.31 ± 0.17 μM, n= 31 for zb_wt, p< 0.01. It also affected the ability of the pump to accelerate the declining phase of the Ca2+ transient trace. It did so more significantly than in the case of single K mutants, as shown by the traces and the histograms of Figure 6B. The half time of the
declining phase was 7.25 ± 1.16 sec, n= 8 for PMCA2zb_K336A; 7.5 ± 0.83 sec, n=6 for PMCA2zb_K338A; 9.37 ± 2.02 sec, n=16 for PMCA2zb_K344A; 6.89 ± 0.93 sec, n=13 for PMCA2zb_K347A; 13 ± 2 sec, n= 16 for PMCA2zb_4M, and. 6.31 ± 0.85 sec, n= 13 for zb_wt, p<0.01) (Figure 7B). The mutations also severely affected the ability of the pump to restore basal Ca\(^{2+}\) levels after cell stimulation, the half time of the peak decay being 11.52 ± 2.27 sec, n= 13 in PMCA2zb_E337A and 11.26 ± 1.73 sec, n= 19 in PMCAzb_S339A, as compared to 6.31 ± 0.85 sec, n= 13 in zb_wt, p<0.01 (Figure 7B).

**Generation, expression and activity of E337A or S339A PMCA2 z/b mutants**

The decision to mutate basic residues (Ks) in the 336-347 domain was dictated by the ability of the domain to bind acidic phospholipids. However, the domain also contains a conserved glutamic acid in position 337 (E337), and a serine in position 339 (S339) (see Figure 4). The **in silico** analysis (see below) suggests that these residues could be involved in polar interactions with other portions of the protein. Thus, they were also mutated. Figure 7A shows that PMCA2zb_E337A and PMCA2 zb_S339A were expressed at levels comparable to those of the transfected PMCA2zb_wt variant and were correctly delivered to the plasma membrane of the transfected cells.

The Ca\(^{2+}\) measurements showed that the PMCA2zb_E337A and the PMCAzb_S339A mutants were less efficient than the PMCA2zb_wt variant in controlling the peak of the Ca\(^{2+}\) transient (1.88 ± 0.19 μM, n= 15 for PMCA2zb_E337A, 1.79 ± 0.25 μM, n= 16 for PMCA2zb_S339A vs. 1.31 ± 0.17 μM, n= 31 for zb_wt, p< 0.01) (Figure 7B). The mutations also severely affected the ability of the pump to restore basal Ca\(^{2+}\) levels after cell stimulation, the half time of the peak decay being 11.52 ± 2.27 sec, n= 13 in PMCA2zb_E337A and 11.26 ± 1.73 sec, n= 19 in PMCAzb_S339A, as compared to 6.31 ± 0.85 sec, n= 13 in zb_wt, p<0.01 (Figure 7B).

**In silico analysis of the two phospholipid binding domains**

Figure 8A shows a cartoon of the PMCA2 in which three of four lysines (K338, K344, K347) contained in the αi domain are predicted to be located approximately at the membrane surface, forming a charged bend which, as already mentioned, could easily accommodate a charged phospholipid head. The electrostatic surface potential of PMCA2 (Figure 8B), shows that the region surrounding the lysines, and stretching towards insertion site A is the only positively charged region in contact with the cytoplasmic side of the membrane. Over thirty residues close to insertion site A could not be modelled, thus, the model is only approximate: however, the missing residues are likely to form a mobile flap extruding from the protein structure. Since conformational switches are required for Ca\(^{2+}\) transport, it could be reasonably suggested that the three lysines would form a binding pocket for initial phospholipid docking. The model agrees well with the experimental findings on the importance of three of the four lysines, as well as with the effect of the 12-residue deletion. Mutation of all four lysines is likely to slow down phospholipid docking, but the positively charged area surrounding insertion site A could partially compensate for this effect.

The two other mutated residues (E337 and S339), are well conserved in PMCA isoforms and in the PMCA across species (see Figure 4). The model positions glutamic acid between two lysines and exposes it to the protein surface, where it could affect other interactions of the pump. As for the serine, its polar group forms hydrogen bond with a glutamine in the α-helix of the pump. As for the serine, its polar group forms hydrogen bond with a glutamine in the α-helix (M3), and with a glutamic acid in the α-helix of domain P (Figure 9). Mutational disruption of hydrogen bonds may have significant structural consequences.

The C-terminal splice variant w/a differs from the w/b variant by a frameshift mutation affecting the second half of the CaM binding region. The model generated by the structural analysis (Figure 10A) shows that the PMCA2 CaM binding region (obtained from the recently deposited NMR structure of the PMCA4 CaM binding region (PDB code: 2KNE) could form an amphiphilic α-helix with a distinctive pattern of charged and hydrophobic residues (30). In the presence of Ca\(^{2+}\) ions CaM folds into a series of α-helices winding around the PMCA2 peptide in a head-to-tail conformation, i.e. the N-terminus of CaM binds the C-terminus of PMCA2. Ca\(^{2+}\) could induce a conformational switch through the stabilization of a stretch of negatively charged residues in a turn conformation, yielding the characteristic collapsed structure of CaM. Interestingly, the final conformation has a strongly negative charge and is stabilized through hydrophobic cages between a benzyl ring and a hydrophobic groove at the center of three CaM α-helices (Figure 10B). In the model, electrostatic attraction is present, but is not crucial to stabilize the final bound conformation. Given the number of charged residues in CaM, electrostatic attraction is likely to initiate the folding process of CaM around the PMCA2 binding region. The substitution of two lysine residues in the CaM binding domain of the w/a variant (see sequence alignment in Figure 10A)
could destabilize the CaM interactions necessary to form the hydrophobic cage for proper binding, explaining the poor sensitivity to CaM of the variant.

Discussion
It would be reasonable to expect that the proximity of site A of alternative splicing to the site that binds acidic phospholipids in the A_L domain could influence the sensitivity of PMCA to acidic phospholipids. The A site insertion could alter the overall conformation of the second cytosolic loop of the pump. It could thus change the spatial connectivity between the phospholipid binding domain and the sequence further upstream, which is involved in the intramolecular inhibitory interaction with the C-terminal calmodulin binding domain. The finding that the A site insert is important for the targeting of PMCA pump to the apical membrane (5) underlines its importance in the general properties of the pump. The role of the A_L phospholipid binding domain has always been obscure, particularly in view of the existence of a second phospholipid binding domain in the C-terminal calmodulin binding sequence (7). One still open question is thus the comparative importance of the two phospholipid binding domains in the regulation of pump activity. Our previous studies on isoform 2 of the PMCA pump had shown differences in the activity of the various A site splicing variants (19,20), showing that the differences in the activity of the various A site insert is important for the targeting of PMCA pump to the apical membrane (5) underlines its importance in the general properties of the pump. The role of the A_L phospholipid binding domain has always been obscure, particularly in view of the existence of a second phospholipid binding domain in the C-terminal calmodulin binding sequence (7). One still open question is thus the comparative importance of the two phospholipid binding domains in the regulation of pump activity. Our previous studies on isoform 2 of the PMCA pump had shown differences in the activity of the various A site splicing variants (19,20), showing that the differences in the activity of the various A site insertion upstream site A through electrostatic attraction.

The measurements of ATPase activity in microsomal membranes of transfected CHO cells have indicated that the w/a variant, as expected, was much less sensitive to CaM than the z/b variant as active as the z/b and w/b isoforms. However, it was also less sensitive to phosphatidylserine, thus underlining the role of the CaM binding domain in the regulation of pump activity by acidic phospholipids. The finding that the z/b and w/b isoforms had the same response to phosphatidylserine had on the other hand indicated that the splicing insertion upstream of the A_L phospholipid binding domain failed to modify the phospholipid sensitivity of the pump.

The analysis of the A_L 12 amino acids lysine-rich stretch, and the model derived from it, had indicated the importance of the conserved lysines in the stretch in the interaction of the phospholipid binding domain with the pump microenvironment. The deletion of the 12 amino acids could for instance directly affect the structure of M3, which is critical to the SERCA pump binding of thapsigargin and could by analogy have special importance to PMCA as well. In the SERCA pump, the segment linking M3 to the A domain is essential for the rotation of the latter and for its correct positioning in the active configuration of the catalytic site (31).

By combining the structural information on the four A_L lysines and on the C-terminal CaM binding region, it could be proposed that the C-terminal domain of the pump that contains the CaM binding region could anchor Ca^{2+} ions to PMCA: it has indeed been shown that Ca^{2+} binding sites are present upstream and downstream the CaM binding domain (32). Once CaM is bound, the PMCA movements could bring the Ca^{2+} ions closer to the lysine containing region near insertion site A through electrostatic attraction.

The finding that the deletion of the 12 residue A_L domain completely abolished pump's activity in the w/a variant, but not in the z/b variant in which it only reduced it, indicated that the activity of the PMCA2 w/a variant strongly depended on the presence of the A_L 380-391 region and, possibly, on the acidic phospholipid binding to it. The finding that the w/a variant was insensitive to PS in the microsomal membranes assay could mean that its stimulation was already maximal under these conditions, as endogenous acidic phospholipids are present in the membranes and could have saturated the PL binding domain. Further addition of PS, thus, could not further stimulate the activity of the w/a variant.

Evidently, CaM activation is not sufficient to make the w/a variant as active as the z/b and the w/b variant. Thus, the difference between the activities of the w/a and z/b variants observed in the measurements performed in intact cells could be related to their interaction with acidic phospholipids, as also suggested by the ATPase activity measurements on microsomal membranes. In other words, the z/b variant would be more active than the w/a variant because of the integrity of its two acidic phospholipid binding sites. The truncation of the protein induced by the site C splicing drastically affected the ability of the pump to bind activator phospholipids and the deletion of 12 residue A_L domain further compromised its activity.
Interestingly, the substitution of all 4 positively charged residues (lysines) reduced the Ca^{2+} extrusion ability of the pump by about the same extent as the replacement of only K344, suggesting a critical role for K344 in pump activity. However, the mutation of two polar residues (E and S) in the same region, affected the pump activity to about the same extent, suggesting that the disruption of the possible interaction of this region of the pump with other pump region (or with other proteins) may be as important to pump activity as the impairment of its ability to bind acidic phospholipids.

References


**FOOTNOTES**

The authors are indebted to Dr. E.E. Strehler (Rochester, MN, USA) for the donation of PMCA2w/a expression plasmid and to Dr. R. Wenthold (Bethesda, MD, USA) for the donation of PMCA2w/a_del12 expression plasmid. The work was supported by the Telethon Foundation (Project GGP04169 to M.B.), the Italian Ministry of University and Research (PRIN 2003 and 2005 to M.B), and by the FP6 program of the European Union (FP6 Integrated Project EUROHEAR, LSHG-CT-2005-512063, to E.C.),
Figure Legends

Figure 1. (A) Linear representation of the alternative splicing options at site A and site C of the PMCA2 transcript. Exons are indicated by shadow boxes, introns by the black line. The numbers in the boxes represent the nucleotide number of each exon. (B) Topography model of the plasma membrane Ca\(^{2+}\)-ATPase and sequences of alternative splicing products of isoform 2. The ten putative transmembrane domains are numbered and indicated by shadow boxes, PL indicates the phospholipid binding domain downstream site A of alternative splicing, D indicates the catalytic aspartate. ATP and CaMBD indicate the ATP binding site and the Calmodulin binding domain, which contains the site C of alternative splicing. (C) Sequences of the PMCA2 region which has been mutated or deleted in the constructs used in this study. The alanine that replaces the mutated residue in the different constructs is indicated in bold. The dashed line represents the 12 amino acids deletion.

Figure 2. Western blotting and densitometric analysis of PMCA2 isoforms overexpression in transfected CHO cells. 20 μg of crude membrane proteins from transfected CHO cells, prepared by a freeze and thaw method, were separated by SDS-PAGE as described in the Experimental procedures section and stained with polyclonal antibody 2N, which recognizes isoform 2 of the pump or against tubulin. The lanes correspond to cells transfected with the indicated variants of PMCA2 fused to GFP. The data are representative of at least 3 independent experiments.

Figure 3. (A) Comparison of Ca\(^{2+}\) transport activity measured on microsomal membranes isolated from CHO cells overexpressing PMCA2 z/b, w/b and w/a variants. Membranes vesicles were preincubated at 37°C and Ca\(^{2+}\) uptake was initiated by the addition of 4 mM ATP (where indicated). 50 μM CaCl\(_2\) was added where indicated. (B) Calmodulin (CaM) dependence of Ca\(^{2+}\) uptake by microsomal membranes preincubated at 37°C with 200 nM CaM. (C) Acidic phospholipids (PS, phosphatidylinerine) dependence of Ca\(^{2+}\) uptake by microsomal membranes preincubated at 37°C with 25 μM PS. In Panels A, B and C the ATPase activity was indicated as the decrease of the absorbance at 340nm. (D) The histograms show the means +/- SD of the activity of the pumps. The activity was expressed as μmolPi/min/μg of protein and calculated as indicated in the Experimental Procedures. The data are representative of at least three experiments with different membranes preparations. * p< 0.05, in respect to the respective controls in the absence of CaM and PS.

Figure 4 ClustalW analysis of the A1 domain and the conservation of mutated residues. The similarity analysis was performed using the ClustalW program. Human PMCA2 sequence (GenBank accession number NP_001674) is listed with other human PMCA isoforms sequences (A) and with those of other species (B). GenBank accession numbers are listed: NP_001001323 (Homo sapiens PMCA1), NP_068768 (Homo sapiens PMCA3), NP_001675 (Homo sapiens PMCA4), XP_509257 (Pan troglodytes PMCA1), AY928176 (Rhesus Macaque PMCA4), NP_036640 (Sus scrofa PMCA2), AAH735643 (Mus musculus PMCA2), BC109173 (Mus musculus PMCA4), Q00804 (Oryctolagus cuniculus PMCA1), NP_777121 (Bos Taurus PMCA1), NP_999517 (Sus scrofa PMCA1), AAK11272 (Rana catesbeiana PMCA2), BC077905 (Xenopus laevis PMCA3), P58165 (Oreochromis mossambicus PMCA2), NP_001116710 (Danio rerio PMCA2), EU559285 (Danio rerio PMCA4), AAR28532 (Procambarus clarkia PMCA3), AAK68551 (Caenorhabditis elegans PMCA3) and AAR13013 (Stylophora pistillata).

Figure 5 (A) Western blotting and densitometric analysis of the variants of the PMCA2 isoform overexpressed in CHO cells. 20 μg of crude membrane proteins from transfected CHO cells, prepared by a freeze and thaw method, were separated by SDS-PAGE as described in the Experimental procedures section and stained with polyclonal antibody 2N. The control lane corresponds to non-transfected cells (CHO). The other lanes correspond to cells transfected with the wt or mutants variants of the PMCA2 pump. The panel also shows the immunocytochemistry analysis of the transfected CHO cells. The immunostaining was carried out with the 2N antibody and revealed with the secondary antibody Alexa Fluor 594. (B) Monitoring of cytosolic [Ca\(^{2+}\)] in CHO cells transfected with cytAEQ and co-transfected with cytAEQ and the wt w/a variant of PMCA2 isoform or deleted PMCA2wa_del12 mutant. (C) Monitoring of cytosolic [Ca\(^{2+}\)] in CHO cells transfected with cytAEQ and co-transfected with cytAEQ and the wt z/b variant of PMCA2 isoform or deleted PMCA2zb_del12 mutant.
The histograms in Panels B and C show the means ±SD of \([\text{Ca}^{2+}]_c\) peaks and of the half time decays from the peaks. The traces are representative of at least 12 independent experiments. *, p<0.01 calculated with respect to control (CHO cells transfected only with cytAEQ).

**Figure 6 (A)** Western blotting and densitometric analysis of the K-mutants of the PMCA2 z/b isoform overexpressed in CHO cells. 20 \(\mu\)g of crude membrane proteins from transfected CHO cells, prepared by a freeze and thaw method, were separated by SDS-PAGE as described in the Experimental procedures section and stained with polyclonal antibody 2N. The control lane corresponds to non-transfected cells (CHO). The other lanes correspond to cells transfected with the wt or mutants variants of the PMCA2 pump. The panel also shows the immunocytochemistry analysis of the transfected CHO cells. The immunostaining was carried out with the 2N antibody and revealed with the secondary antibody Alexa Fluor 594. (B) Monitoring of cytosolic \([\text{Ca}^{2+}]_c\) in CHO cells transfected with cytAEQ and co-transfected with cytAEQ and the PMCA2zb_K336A, PMCA2zb_K338A, PMCA2zb_K344A, PMCA2zb_K347A, or PMCA2zb_4M, alternatively. The histograms show the means ±SD of \([\text{Ca}^{2+}]_c\) peaks and of the half time decays from the peaks. The traces are representative of at least 12 independent experiments. *, p<0.01 calculated with respect to PMCA2zb_wt (CHO cells transfected with wt PMCA2 z/b pump).

**Figure 7 (A)** Western blotting and densitometric analysis of the E and S-mutants of the PMCA2 z/b isoform overexpressed in CHO cells. 20 \(\mu\)g of crude membrane proteins from transfected CHO cells, prepared by a freeze and thaw method, were separated by SDS-PAGE as described in the Experimental procedures section and stained with polyclonal antibody 2N. The control lane corresponds to non-transfected cells (CHO). The other lanes correspond to cells transfected with the wt or mutants variants of the PMCA2 pump. The panel also shows the immunocytochemistry analysis of the transfected CHO cells. The immunostaining was carried out with the 2N antibody and revealed with the secondary antibody Alexa Fluor 594. (B) Monitoring of cytosolic \([\text{Ca}^{2+}]_c\) in CHO cells transfected with cytAEQ and co-transfected with cytAEQ and the PMCA2zb_E337A or the PMCA2zb_S339A. The histograms show the means ±SD of \([\text{Ca}^{2+}]_c\) peaks and of the half time decays from the peaks. The traces are representative of at least 12 independent experiments. *, p<0.01 calculated with respect to PMCA2zb_wt (CHO cells transfected with wt PMCA2 z/b pump).

**Figure 8 (A)** Overview of the PMCA2 model, shown in cartoons and colour-coded for the different canonical domains, with the four mutated lysines highlighted as red spheres. The approximate location of the membrane limits are shown with lines and the third transmembrane helix is labelled as M3. Note that the C-terminal part of PMCA2 from residue 1088 onwards could not be modelled. Insertion site A is highlighted. (B) Electrostatic potential of the PMCA2 accessible surface. The structure is shown in the same orientation as in Panel A and rotated around the central axis (right). The location of the mutated lysine residues is circled. Note how the area around between the four lysines and insertion site A is the only PMCA2 region with positive potential in contact with the membrane.

**Figure 9** Representation of the two residues, S337 (pink) and E339 (blue). These are shown as sticks and yellow dashed lines indicate interatomic contacts or hydrogen bonds with neighbouring residues.

**Figure 10 (A)** Structural model of the calmodulin-binding region of PMCA2 (top) and relative sequence alignment (bottom). The amphipathic PMCA2 helix is shown in grey at the center of the structure, with residues in purple and pink defining the N- and C-terminal motifs. The calmodulin structure is shown with progressively varying colour, from blue (N-) to red (C-terminus). \(\text{Ca}^{2+}\) ions are shown as green spheres. The sequence alignment shows the structural template (PMCA4, PDB code 2KNE) together with two PMCA2 variants. The last line defines the sequence motif for calmodulin-binding. Note how PMCA2 w/a lacks two crucial lysine residues for the second motif. (B) Electrostatic surface of the calmodulin-binding region of PMCA2 with bound CaM in the same orientation as in Panel A.
**Figure 1**

**A**

![Diagram showing site A and site C with w, a, and b labels]

**B**

- **Site A**: EEEKKDKKGKGGDK&DGLPLASADGAASNADAASANASLSLVNGKMQDGLYNYDQSQSKAKQDDGA A
  - **w insertion (45aa)**

- **Site C**

**Variant a**: GLNRIGYQ/HYVYNT/KSGASF/QGALRQ/QWTS/QSQD/VANSSLSPRSV/LSNALSS PTSLPPAAAG/HPREGVIP* 1174 aa

**Variant b**: GLNRIGYQ/HYVYKAFRSSLVEGKPESTSHNFAMHEPEF/ESQPH/PL/I/D/D/T D LEEDAALK/QSSPPSSLNKSALDISG/INLTTDYSKS/PATSSPG/SRLCTSL* 1210 aa

**C**

- **PMCA2_del12**: ASMHK
- **PMCA2_K336A**: ASMHKAESVLQGLTCLAV
- **PMCA2_K338A**: ASMHKKEASVLQGLTCLAV
- **PMCA2_K344A**: ASMHKKEASVLQGLTCLAV
- **PMCA2_K347A**: ASMHKKEASVLQGLTALAV
- **PMCA2_4M**: ASMHKKEASVLQALTV
- **PMCA2_E337A**: ASMHKKAESVLQGLTCLAV
- **PMCA2_S339A**: ASMHKKEAASVLQGLTCLAV
Figure 4
Figure 5
Figure 6
**Figure 7**

A

![Western Blot](image)

**Relative Expression**

- PMCA2
- PMCA2 \(\text{E337A}\)
- PMCA2 \(\text{S339A}\)

Bars represent average expression levels with error bars indicating standard error. 

- PMCA2 \(n=3\)
- PMCA2 \(\text{E337A} n=3\)
- PMCA2 \(\text{S339A} n=3\)

B

**[Ca^2+]_i (µM) vs Time (sec)**

- PMCA2 \(\text{E337A}\)
- PMCA2 \(\text{S339A}\)

Graphs show changes in intracellular calcium levels following ATP and CaCl_2 stimulation.

ATP 100 µM
CaCl_2 1mM
Figure 8
Deletions and mutations in the acidic lipid-binding region of the plasma membrane Ca$^{2+}$ pump: a study on different splicing variants of isoform 2
Marisa Brini, Francesca Di Leva, Claudia K. Ortega, Teuta Domi, Denis Ottolini, Emanuela Leonardi, Silvio C.E. Tosatto and Ernesto Carafoli

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