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**Functional properties of an unusual isoform of the
plasma membrane calcium ATPase: PMCA 2**

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A papá

*Hay bajo el sol un momento para todo,
y un tiempo para hacer cada cosa:
Tiempo para nacer, y tiempo para morir;
tiempo para plantar, y tiempo para arrancar lo plantado;
tiempo para matar y tiempo para curar;
tiempo para demoler y tiempo para edificar;
tiempo para llorar y tiempo para reír;
tiempo para gemir y tiempo para bailar;
tiempo para lanzar piedras y tiempo para recogerlas;
tiempo para los abrazos y tiempo para abstenerse de ellos;
tiempo para buscar y tiempo para perder;
tiempo para conservar y tiempo para tirar fuera;
tiempo para rasgar y tiempo para coser;
tiempo para callarse y tiempo para hablar;
tiempo para amar y tiempo para odiar;
tiempo para la guerra y tiempo para la paz*

SUMMARY

The plasma membrane calcium ATP-ase (PMCA) represents a primary system for the specific extrusion of calcium from eukaryotic cells. Together with Na⁺/Ca²⁺ exchangers, it is the major plasma membrane transport system responsible for the long-term regulation of the resting intracellular calcium concentration. The PMCA contains 10 membrane-spanning segments and the NH₂ and COOH termini are both located on the cytosolic side of the membrane.

The PMCA pump is the product of 4 separate genes, isoform diversity being further increased by a complex pattern of alternative splicing of the primary transcripts. The four basic gene products (PMCA 1-4) and the numerous splice variants vary in expression level during development, have peculiar distribution in tissues and within cells, and differ with respect to functional parameters, especially those involving regulation properties. PMCA 1 and 4 are ubiquitously expressed while 2 and 3 are mostly found in the central nervous system and, in lesser amounts, in the striated muscle.

Alternative splicing is peculiarly complex in PMCA2 because it involves the insertion of up to three novel exons at site A (variant *w*) and of two at site C (variant *a*). The insert at site C creates instead a novel stop codon, leading to the truncation of the pump. The site-C insertions eliminate approximately half of the calmodulin binding domain; those at site A occur next to a domain that binds activatory acidic phospholipids.

Calcium enters the stereocilia of hair cells through mechano-electrical transduction channels opened by the deflection of the hair bundle and is exported back to endolymph by an unusual splicing isoform (*wa*) of plasma-membrane calcium-pump isoform 2 (PMCA2).

Ablation or missense mutations of the pump cause deafness, as described for the G283S mutation in the deafwaddler (*dfw*) mouse. A deafness-inducing missense mutation of PMCA2 (G293S) has been identified in a human family. The family also was screened for mutations in cadherin 23, which accentuated hearing loss in a previously described human family with a PMCA2 mutation. The *wa* variant and their mutations were overexpressed in Sf9 cells. At variance with the other PMCA2 isoforms, it became activated only marginally when exposed to a Ca²⁺ pulse. The defect is more pronounced in the *dfw* mutant than in the G293S (human) mutant. A third mutation was analyzed:

Oblivion mouse mutation (where a Serine is replaced by a Phenylalanine at position 877 in TM6), it was slightly more active than the pumps bearing the 283 and 293 mutations but still much lower than that of the wild type *wa*.

The other important aspect studied, it is the regulation mechanism of the PMCA 2 *wa* variant. Microsomal membranes isolated from CHO cells transfected with PMCA 2 *zb*, PMCA 2 *wb*, PMCA 2 *wa* and PMCA 2 *wa* (Tommy), where a mutation in the ATP binding site occurs in the valine 586, were assayed. Both PMCA 2 *zb* and PMCA 2 *wb* had higher basal activity than PMCA 2 *wa*. PMCA 2 *wa* (Tommy) only had about 25 per cent of the activity of PMCA 2 *wa*. In the presence of Calmodulin, PMCA2 *zb* and *wb* showed the highest response to it i.e., the activity of these isoforms was over eight times higher than of PMCA 2 *wa*. PMCA2 *wa* had lower affinity for calmodulin than PMCA2 *zb* and *wb*, but had still higher affinity than the corresponding Tommy mutant. The mechanism by which different phospholipids activate the PCAs is not known. However, given the location of the lipid-binding sequences in the pump, one speculated that the interaction of acidic phospholipids with the calmodulin binding domain leads to some structural rearrangement that weakens the autoinhibitory intramolecular interactions formed by the COOH-terminal tail. The PMCA 2 *zb* and *wb*, when overexpressed in CHO cells, had the same response to this acidic phospholipids implying that the inserts next to the N-terminal phospholipids binding domain (variant *w*) was not disturbed by the splicing insert. The PMCA *wa* was absolutely not stimulated by phosphatidyl serine apparently, then, no activation by acidic phospholipids can occur when the C-terminal binding site is undisturbed. The response of the PMCA 2 *b* pumps to PS was 4 fold higher than that of the *wa* variant.

To investigate whether Calmodulin indeed removes its binding domain from the cytosolic loops of the pump, we have introduced a mutation in one of the two sites that interact with the CaM binding domain in the cytosolic loops of the pump. Prior to this, the PMCA pump was modeled to decide the residue that should have been mutated. From the model and previous works we decided to mutate the methionine 265 in Alanine (M265A). The activity of the PMCA 2 *zb* and M265A pumps expressed in transfected CHO cells evaluated as a function of Ca^{2+} in both the presence and absence of calmodulin shows that the M265A mutant has a response to calmodulin which was the same as that of the wild type at saturating concentrations of calmodulin. The activation by CaM was higher in the M265A pump with respect to wild type pump at 1nM, 5nM and 15 nM calmodulin concentrations. This suggests that the autoinhibitory

sequence was bound with less affinity to the intramolecular binding site in the Met265 mutant as less calmodulin was required to relieve the inhibition. Evidently, the inhibition of Met 265 decreased the affinity of the C-terminal autoinhibitory domain, making it easier for calmodulin to remove it from binding site.

RIASSUNTO

Le Ca^{2+} -ATPasi della membrana plasmatica (PMCA) sono responsabili dell'espulsione specifica del Ca^{2+} dal citoplasma nelle cellule eucariotiche. Assieme allo scambiatore $\text{Na}^+/\text{Ca}^{2+}$ rappresentano il meccanismo di trasporto più importante per mantenere la concentrazione del Ca^{2+} intracellulare a livelli basali. Le pompe PMCA contengono 10 domini transmembrana e presentano le estremità amminoterminali e carbossiterminali rivolte verso il citosol.

Nelle cellule eucariotiche sono presenti 4 diverse isoforme di PMCA, denominate PMCA1, 2, 3, 4 e codificate da geni distinti. Il numero delle isoforme è ulteriormente aumentato da meccanismi di *splicing* alternativo del trascritto primario di ciascuno di questi geni. Le quattro isoforme e le numerose varianti di *splicing* mostrano un livello di espressione che varia durante lo sviluppo embrionale, una distribuzione tessuto-specifica e presentano caratteristiche biochimiche e funzionali proprie. Le isoforme 1 e 4 sono ubiquitarie mentre le isoforme 2 e 3 sono prevalentemente espresse nel sistema nervoso centrale e a livelli minori nel muscolo scheletrico.

Lo *splicing* alternativo è un processo abbastanza complesso nella PMCA 2 perché richiede l'inserzione di tre nuovi esoni nel sito A (variante *w*) e di due nel sito C (variante *a*). L'inserzione nel sito C genera un nuovo codone di stop, provocando la troncatura della pompa. Le inserzioni al sito C eliminano approssimativamente metà del dominio che lega la calmodulina; invece quelli nel sito A, sono prossimi al dominio che lega i fosfolipidi acidi.

Il calcio entra nelle stereocilie delle cellule ciliate attraverso l'apertura dei canali di meccanotrasduzione grazie alla deflessione della stereocilia ed è esportato alla endolinfa attraverso di una variante di *splicing* particolare (*wa*) della Ca^{2+} -ATPasi della membrana plasmatica 2 (PMCA 2).

Ablazioni e mutazioni missense della pompa provocano sordità, come è descritto per la mutazione G283S nel topo deafwaddler (*dfw*). Una mutazione missense della PMCA 2 (G293S) che induce sordità è stata identificata in una famiglia umana. La famiglia fu esaminata anche per mutazioni nella caderina 23, la quale accentuò la perdita dell'udito nella famiglia previamente descritta associata alla mutazione della PMCA 2. La variante *wa* e sui mutanti furono sovraespresse in cellule Sf9. A differenza delle altre varianti della PMCA, *wa* è attivata solo marginalmente quando è sposta a polsi di calcio. L'effetto è ancora più evidente nel mutante *dfw* che nel mutante umano G293S. Una

terza mutazione fu analizzata: la mutazione del topo denominata Oblivion (dove una serina è sostituita per una fenilalanina nella posizione 877 nel dominio di transmembrana 6), questa fu leggermente più attiva delle pompe che portano le mutazioni 283 e 293 però ancora molto più basso di quelle del wild type *wa*.

Un altro aspetto importante studiato, è il meccanismo di regolazione della variante PMCA 2 *wa*. Membrane isolate di cellule CHO trasfettate con PMCA 2 *zb*, PMCA 2 *wb*, PMCA 2 *wa* e PMCA 2 *wa* (Tommy), dove apparve una mutazione nel sito di legame del ATP nella valina 586 furono studiate. Tanto PMCA 2 *zb* e PMCA 2 *wb* hanno un'attività basale più alta in confronto alla variante PMCA *wa*. PMCA 2 *wa* Tommy ha soltanto il 25 per cento di attività in confronto alla variante *wa*. In presenza di calmodulina, PMCA 2 *zb* e PMCA 2 *wb* mostrano una risposta più elevata, l'attività di queste varianti fu di otto volte maggiore a quella della variante *wa*. PMCA 2 *wa* ha una bassa affinità per la calmodulina probabilmente basata nel fatto che manca di metà del dominio che la lega. L'affinità per il calcio della variante *wa* è comunque più alta a quella del mutante Tommy.

Il meccanismo mediante il quale diversi fosfolipidi attivano le PMCA non è ancora noto. Tuttavia, data la localizzazione delle sequenze che legano i lipidi nella pompa, si può speculare che le interazioni dei fosfolipidi con il dominio che lega la calmodulina inducono cambiamenti strutturali che indeboliscono le interazioni intramolecolari con la porzione C-terminale della pompa. PMCA 2 *zb* e PMCA 2 *wb* hanno la stessa risposta ai fosfolipidi acidi significando che l'inserzione prossima al dominio che legano i fosfolipidi (variante *w*) non furono disturbate per le inserzioni di splicing. La PMCA 2 *wa* non fu assolutamente stimolata per la fosfatidilserina (PS), per tanto, la non attivazione può verificarsi forse, quando il sito di legame C-terminale è indisturbato. La risposta delle varianti *b* alla PS fu quattro volte in confronto alla variante *wa*.

Per investigare se la Calmodulina davvero rimuove il suo sito di legame dai loops citoplasmatici della pompa, abbiamo introdotto una mutazione in uno dei siti che interagiscono con il dominio che lega calmodulina che agisce inibendo la pompa. Prima di questo, la PMCA fu modellata per decidere che residuo doveva essere mutato. Dai risultati del modello e di lavori precedenti, fu deciso di mutare la metionina 265 in alanina. L'attività della pompa wild type (PMCA 2 *zb*) e del mutante (M265A) fu analizzata in presenza e assenza di calmodulina. A concentrazioni saturanti di calmodulina tutti e due le pompe hanno la stessa risposta al nominato attivatore. A bassa concentrazione di calmodulina (1nM, 5nM e 15 nM), il mutante sembra essere più

attivo del wild type. Questo potrebbe suggerire che la sequenza che inibisce la pompa se lega con una minore affinità nel mutante e una concentrazione minore di calmodulina e necessaria per liberare la sequenza che lega la calmodulina ai siti di rispettivi di legame. Evidentemente l'inibizione della M265 diminuisce l'affinità al dominio autoinibitorio C-terminale.

TABLE OF CONTENTS

ABBREVIATIONS	14
INTRODUCTION.....	16
1- General principles of Ca ²⁺ signaling.....	18
2- Ca ²⁺ sensors	19
3- Ca ²⁺ -transporting protein	21
4- The plasma membrane calcium pump(PMCA)	27
4.1- Isolation and purification of the Calcium Pumps	28
4.2-Cloning of the pumps and recognition of the isoforms	29
4.3- The Plasma Membrane Calcium Pumps:	
Structural and Regulatory Characteristics	32
4.4- Isoforms of the PMCA pump	33
4.5- Splicing variants	36
4.6- Protein interactors of PMCA pumps.....	39
4.7. PMCA pump and diseases	40
4.7.1- PMCA pump knock out mice	40
4.7.2- Heredity deafness and other diseases conditions	42
EXPERIMENTAL PROCEDURES	44
1- Baculovirus Expression System	44
1.1- Baculovirus Expression vectors.....	45
1.2- Cytopathogenesis.....	45
1.3- Generating recombinant virus by Homologous Recombination.....	46
1.4- Generating recombinant virus by site-specific transposition.....	47
2- Cloning and virus generation of PMCA <i>zb</i> and <i>wa</i> variants.....	48
2.1- Construction of the recombinant baculovirus.....	49
2.2- Plasmid DNA isolation from bacteria. Miniprep.....	49
2.3- Plasmid DNA isolation from bacteria. Maxiprep.....	50
2.4- DNA digestion and agarose gel electrophoresis	50
2.5- DNA fragments purification from agarose gel	51
2.6- DNA ligation	51
2.7- Transformation of competent <i>E.coli</i> cells.....	51
2.8- DNA sequencing.....	51
2.9- Generation of Recombinant Bacmid.....	52
2.10- Transformation of DH10Bac <i>E.coli</i> cells	52
2.11- Analysis of recombinant DNA by PCR.....	54
2.12- Transfection of insect cells	55
2.13- Virus amplification	56
3- Expression and purification of PMCA 2 <i>zb</i> and <i>wa</i> variants.....	56
3.1- Infection and expression of PMCA 2 in insect cells.....	56
3.2- Sf9 cell membrane preparation	57
3.2- Solubilization of PMCA 2 proteins	58
3.4- Calmodulin Affinity Chromatography	58
3.5- His-Tag affinity chromatography	58
3.6- PMCA 2 preparation for SDS-PAGE analysis	59

3.7- PMCA 2 Western blotting analysis	59
3.8- Western blotting with antiCalmodulin antibody.....	61
4- Expression of PMCA pumps in CHO cells	61
4.1- Continuous cell lines.....	61
4.2- Transfection	62
4.3- Isolation of microsomes from CHO cells	62
4.4- Mutagenesis	63
5- ATPase activity.....	63
6- Immunocytochemistry	64
7- Computational methods	65
7.1- Sequence alignment	65
7.2- Structural model.....	65
RESULTS	66
I. Expression and purification of PMCA 2 pump	66
1- Expression of the PMCA 2 isoforms in Baculovirus system	67
1.1- Localization of PMCA 2 <i>zb</i> in insect cells	67
1.2- Expression and purification of PMCA2 <i>zb</i>	68
1.3- Time course of PMCA 2 <i>zb</i> expression in Sf9 cells	68
1.4- Preparation of Sf 9 cells membranes for purification with a calmodulin resin	70
1.5- PMCA 2 <i>zb</i> solubilization and purification by a calmodulin affinity resin	70
1.6- Construction of a Recombinant Baculovirus with a His-Tag Vector.....	72
1.7- Time course PMCA 2 <i>wa</i> expression in Sf9 cells.....	73
1.8- Sf 9 insect cells membrane preparation for His-Tag affinity purification	74
1.9- PMCA 2 <i>zb</i> and <i>wa</i> purification by His-Tag affinity column.....	75
II. Biochemical study of mutants of plasma membrane calcium pump isoform 2 causing deafness.....	77
1- Construction and expression of PMCA 2 <i>wa</i> mutants.....	78
1.2- Determination of the Ca ²⁺ transport activities of microsomes from PMCA 2 <i>zb</i> , PMCA2 <i>wa</i> and of mutated pumps of the <i>wa</i> variant	78
1.3- Study of a novel mutation near to the ATP binding domain of the PMCA 2 pump	80
III. Functional properties of the PMCA 2 <i>wa</i> splice variant.....	83
1- Functional studies of PMCA <i>wa</i> . Unusual variant of PMCA 2 pump.....	84
1.1- Expression of PMCA 2 splicing variants in CHO cells.....	84
1.2- Determination of Ca ²⁺ transport activities of Microsomes from CHO transfected CHO cells with PMCA <i>zb</i> , <i>wb</i> , <i>wa</i> and Tommy.....	84
1.3- Calmodulin activation.....	85
1.4- Activation by Phosphatidyl-serine.....	86

IV. Structural Model of PMCA 2 and biochemical study of the effect of a Met265Ala mutation in one of the domain that mediate the autoinhibition of the pump	88
1- Comparative model between SERCA and PMCA 2	88
1.1-Functional state of the PMCA 2 <i>zb</i> and M265A mutant.....	92
DISCUSSION	96
REFERENCES.....	102

ABBREVIATIONS

AA	Aminoacid
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumine
cDNA	Complementary DNA
C ₁₂ E ₈	Octaethyleneglycol mono-n-dodecylether
O.D.	Optical density
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GAL	Galactose
HEPES	4-(2-hydroxyethyl)-piperazineethanesulfonicacid
His (H)	Histidine
IP ₃	Inositol 1,4,5-trisphosphate
IP ₃ R	Inositol 1,4,5-trisphosphate receptor
IPTG	Isopropyl 1-thio-β-D-galactopyranoside
Kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani
LDH	Lactate Dehydrogenase
MOPS	3-(N-morpholino)propane-sulfonic acid
NADH	Nicotinamide adenine dinucleotide
NCX	Na ⁺ /Ca ²⁺ exchanger
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PEP	Phosphoenolpyruvate

PK	Pyruvate Kinase
PMCA	Plasma membrane Ca ²⁺ ATP-ase
PMSF	Phenylmethanesulphonyl fluoride
PS	Phosphatidyl Serine
PVDF	Polyvinylidene Fluoride
RyR	Ryanodine receptor
SR	Sarcoplasmic reticulum
SERCA	Sarco/endoplasmic reticulum Ca ²⁺ -ATPase
SDS	Sodium dodecyl sulfate
Sf9	<i>Spodoptera frugiperda</i> insect cells
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
Triton X-100	Octyl phenol ethoxylate
TWEEN 20	Polyoxyethylene (20) sorbitan monolaurate

INTRODUCTION

The evolutionary transition from unicellular to multicellular life brought with it the division of labor among cells. This in turn generated the necessity of exchanging signals among the cells that formed the new organisms: as is well known, as a rule monocellular organisms do not depend on the exchange of signals, their mutual interactions being largely confined to the exchange of nutrients. Cell signaling is thus essentially a hallmark of pluricellular organisms, in which a multiplicity of external messages, carried by numerous *first messengers*, is translated inside the cells into specific responses mediated by a less numerous group of newly produced chemicals, *the second messengers*.

External signal, e.g. hormones, had long been known, but second messengers have only arrived on the scientific scene more recently, with the discovery of cAMP in the 1950s. One of the second messengers, however, had become known much earlier even if the significance of the discovery was only appreciated much later, once the importance of the observation became obvious: it was calcium (Ca^{2+}), which was the main actor in the experiment published by Ringer in 1883 (1)

The advancement of knowledge rapidly became all-encompassing: as a large amounts of new information was gathered, it gradually became clear that Ca^{2+} had a number of properties that made it unique among all other carriers of biological information. Three of these properties are particularly striking. The first is the ability to act both as a first and as a second messenger. The second is the autoregulative property of the Ca^{2+} signal, i.e., Ca^{2+} itself may control the generation and the regulation of the information it carries. Finally, and perhaps most importantly, the Ca^{2+} signal has a clear ambivalent character: although essential to the correct functioning of cells, it can easily become a messenger of death. (Fig. 1)

Signaling operations typically involve the interaction of messenger chemicals with the surface of target cells, which then process the incoming message and initiate an internal signaling chain which is normally mediated by soluble signaling molecules. This chain of events is the most common way of exchanging information between cells but is by no means unique. Cells can also communicate with each other through direct contacts in the form of gap junctions or through surface proteins that recognize protein counterparts on the surface of other cells. As for first messengers, some may by pass the plasma membrane and penetrate directly into target cells. Once there, they interact with

receptors in the cytoplasm and/or the nucleus and act without the intermediation of second messengers. These alternative possibilities are all interesting and important: however, the classical way of exchanging information remains that based on the interaction of first messengers with plasma membrane receptors and on the generation of diffusible second messengers inside cells. In considering Ca^{2+} from this perspective, a number of peculiarities emerges that are difficult to reconcile with the canonical second messenger concept. Ca^{2+} certainly behaves as a diffusible second messenger generated (liberated) inside cells in response to the interaction of a number of first messengers with plasma membrane receptors. However, the increase of cell Ca^{2+} is not directly linked to the processing of first messenger signals, as is the case, for instance, for cAMP. It occurs one step further down the signaling chain and follows the generation of another second messenger, e.g., InsP_3 , which will then liberate Ca^{2+} from intracellular stores. Strictly speaking, then, Ca^{2+} should be defined as a third messenger. But Ca^{2+} can also penetrate directly into cells from the external spaces to initiate the intracellular signaling chain. This, however, is not unique to Ca^{2+} : other second messengers, e.g., the NO radical, may also have this dual origin (interstitial spaces and intracellular ambient).

The peculiarities above may be considered minor and essentially formal. Another distinctive property of the Ca^{2+} signal, however, is more substantial. Ca^{2+} has the ability, now being documented in a growing number of cell types, to recognize specific G-protein-linked plasma membrane receptors. Following the interaction, a G-protein-mediated chain of events is initiated that ends in the modulation of the release of Ca^{2+} itself from intracellular stores. The finding that Ca^{2+} acted on a plasma membrane receptor as any other canonical first messenger came as a surprise **(2)**: the concentration of first messengers is very low and necessarily fluctuates around cells, whereas that of Ca^{2+} is high and remains remarkably constant. Thus, the possibility that extracellular Ca^{2+} would act as a first messenger had not been generally considered. However, it had long been known that cells secreting the calciotropic hormones that regulate the homeostasis of Ca^{2+} in the organism, e.g., the cells of the parathyroid gland, modify the release of the hormones in response to perturbations of external Ca^{2+} . Indications that the mechanism by which these cells sensed the changes in external Ca^{2+} may have involved a plasma membrane receptor came recently. The first clear findings in this direction were the discoveries that raising the level of external Ca^{2+} induced a transient

and then a sustained elevation of Ca^{2+} in parathyroid cells [12], which was linked to the activation of phospholipase C and thus the generation of InsP_3 . (3)

1- General principles of Ca^{2+} signaling

Eukaryotic cells are surrounded by media containing free Ca^{2+} concentrations that exceed 1mM, but manage to maintain a free Ca^{2+} concentration in the cytoplasm that is four orders of magnitude lower. This very low internal concentration is dictated by the necessity to avoid the precipitation of Ca^{2+} , given the poor solubility of Ca^{2+} -phosphate salts. It also prevents the prohibitive expenditures of energy that would otherwise be necessary to significantly change the background concentration of Ca^{2+} in the vicinity of the targets that must be regulated: if the resting free Ca^{2+} in the cytosol were much higher than nM, considerably larger amounts of energy would have to be invested to change it substantially. The total Ca^{2+} concentration within cells is naturally much higher than nM but is reduced to the sub- μM ionic range first by binding to membrane (acidic) phospholipids, to low molecular weight metabolites, to inorganic ions like phosphate, and then by complexation to specific proteins.. These belong to several classes: one comprises the membrane intrinsic proteins that operate as Ca^{2+} transporters in the plasma membrane and in the membranes of the organelles. These proteins play the most important role in the maintenance of the cellular Ca^{2+} homeostasis as they move Ca^{2+} back and forth between the cytosol, where most of the targets of the Ca^{2+} signal are located, and the extracellular spaces or the luminal spaces of the organelles. Depending on whether large amounts of Ca^{2+} must be moved, or whether only the fine-tuning of Ca^{2+} down to very low concentration levels is required, low or high Ca^{2+} affinity transporters become active.

The low-molecular compounds that have propensity to complex Ca^{2+} (e.g., phosphates, ATP, aminoacids, acidic phospholipids of membranes) do so with low affinity, and also easily complex other ions (e.g. Mg^{2+}) that may be more concentrated in the intracellular environment. To achieve Ca^{2+} specificity, evolution has developed complex molecules able to exploit the special chemistry of Ca^{2+} by offering to it specific binding sites. (4)

The ability of these sites to efficiently bind Ca^{2+} even in the greatly reduced ionic concentration range resulting from the low affinity binding to the compounds mentioning above, and to do so in the presence of much higher concentration of the other cations, is made possible by the coordination flexibility of Ca^{2+} .(5) The

combination of charge and dimension of this ion makes it an ideal ligand for irregularly shaped binding cavities, where the distance to the coordinating oxygens may vary greatly. This could be convenient compared to Mg^{2+} , whose ionic radius is much smaller than that of Ca^{2+} and whose polarizing power much greater.

The conclusion that emerges is that the advantages of Ca^{2+} as a ligand for complex (protein) molecules made it a particularly attractive candidate, perhaps even an obligate one, as a messenger that would carry information to the intracellular ambient.

One final point must be mentioned before closing the discussion of the advantages of Ca^{2+} : The energetic metabolism of cells is phosphate-oriented: ATP is the universal energetic currency, and it is self evident, given the propensity of Ca^{2+} phosphate salts to precipitate, that high concentrations of the ATP cleavage product phosphate in the cell ambient are only became possible if means were made available to keep the Ca^{2+} concentration very low. Evolution has evidently exploited the favorable properties of Ca^{2+} as a ligand to develop means to efficiently lower its concentration to a point where there would be no detrimental consequences for phosphate-oriented energetic metabolism.

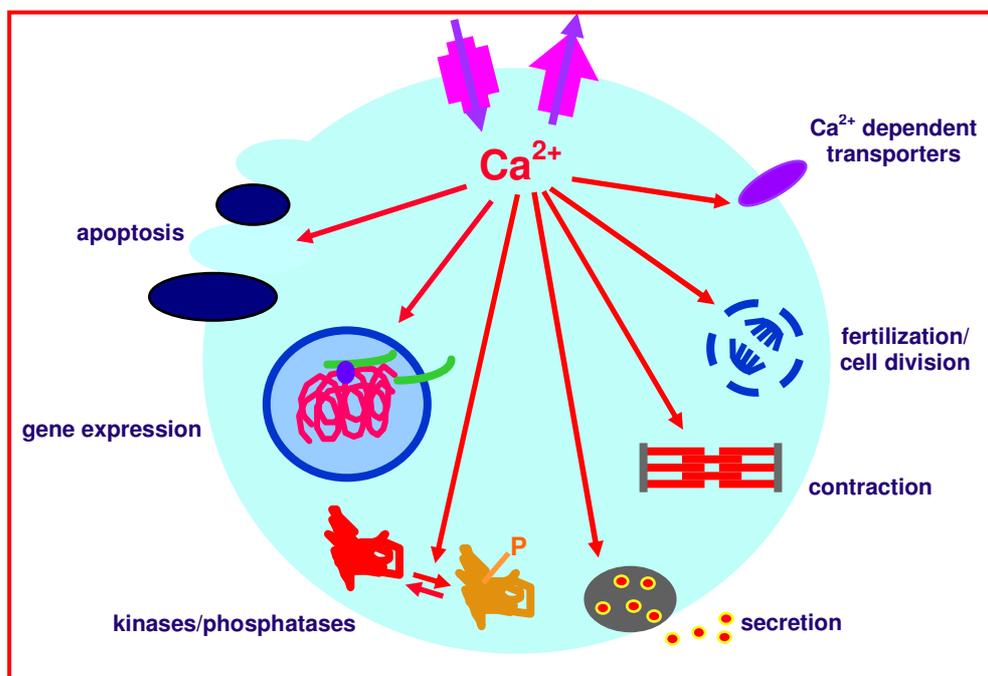


Figure 1: Ca^{2+} - modulated functions in mammalian cells

2- Ca²⁺ sensors

EF-hand proteins are the most important and best-characterized Ca²⁺ sensors. They bind Ca²⁺ using helix–loop–helix motifs (there are between 2 and 12 such motifs in the various EF-hand proteins), which ligate Ca²⁺ to the side chain and carbonyl oxygen atoms of some invariant residues. Variations in the coordination pattern have been described. For example, oxygen atoms from neighboring helices can ligate Ca²⁺. Other Ca²⁺ sensors — for example, the annexins, gelsolin and proteins that contain C2 domains (a compact β -sandwich of two four-stranded β -sheets) — should also be mentioned, even if their role is not as general or as well understood as that of the EF-hand proteins. Ca²⁺ sensors normally modulate target enzymes that are not Ca²⁺ sensors themselves, but there are enzymes that can be modulated by Ca²⁺ directly, that is, they are genuine Ca²⁺ sensors.

The founder member of the EF-hand protein family was the Ca²⁺-buffering protein parvalbumin, as mentioned above. Its crystal structure was solved by Kretsinger and Nockholds (6) in 1973, and its basic Ca²⁺-binding principles were extrapolated to other proteins, the tertiary structures of which only became available much later. The family of EF-hand proteins has now grown to include hundreds of members, the most thoroughly investigated of which is calmodulin.

Most of what we now know about the processing of Ca²⁺ signals by EF-hand proteins came from studying calmodulin. Ca²⁺ induces a conformational change that does not affect the overall shape of calmodulin, but that makes its surface more hydrophobic (Fig. 2). Calmodulin then approaches its targets, collapses around their binding domains and transmits Ca²⁺ information to them. Ca²⁺-free calmodulin (apocalmodulin) can also bind proteins through several different binding motifs (such as an IQ motif, which is a consensus sequence of about 10 residues that begins with isoleucine-glutamine) and may have an effect also in the absence of Ca²⁺. The proteins that are modulated by apocalmodulin include enzymes (for example, phosphorylase kinase and some unconventional myosins), structural proteins and membrane proteins (such as receptors and ion channels) (7). As a modulator, calmodulin is 'uncommitted', that is, it interacts with numerous targets and is associated with them reversibly. However, calmodulin can also become permanently associated with some proteins; for example, this is the case for the inducible form of nitric oxide synthase. Perhaps, calmodulin modulation is so important to some enzymes that, once they have bound it, they never release it.

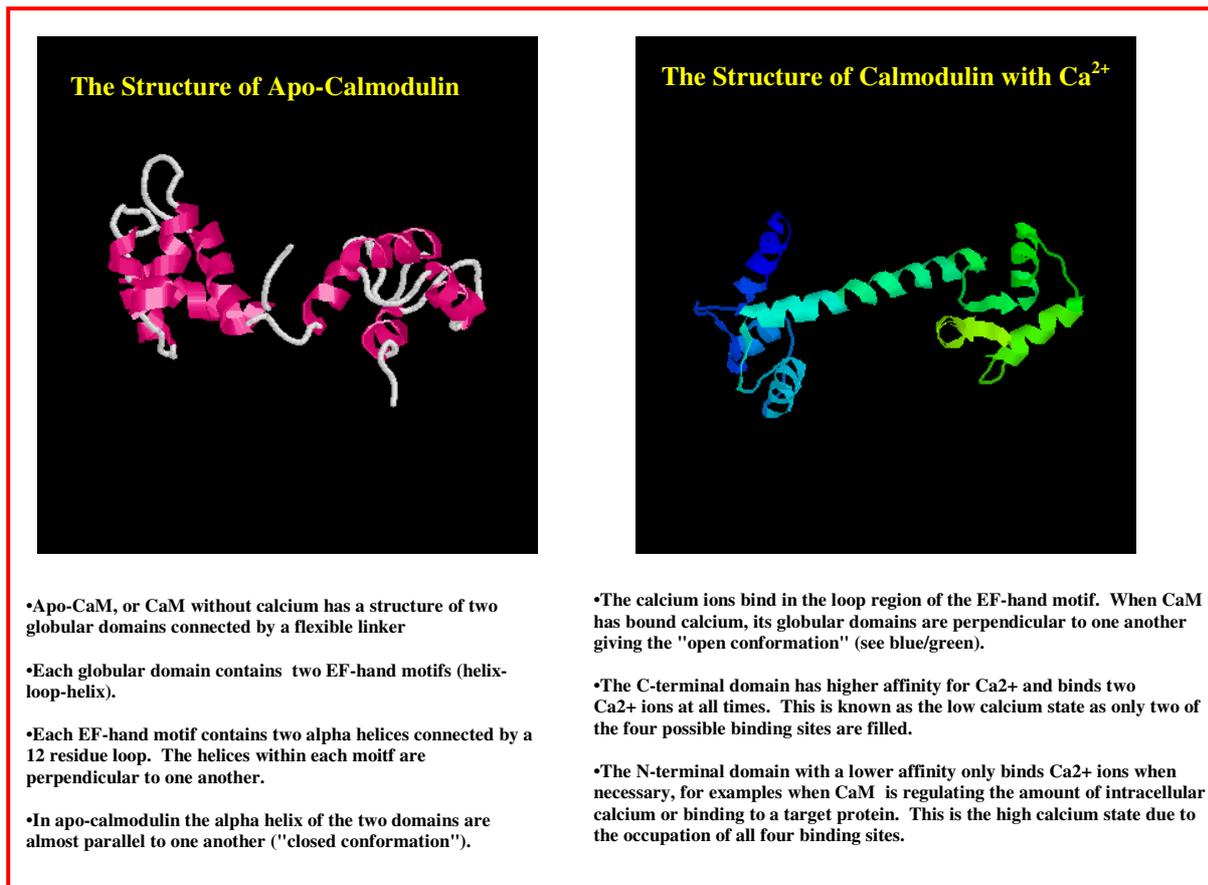


Figure 2: Conformational changes of Calmodulin (CaM) structure

Interestingly, enzymes have now been described that contain their own 'calmodulin', either as a separate subunit, as is the case for the Ca²⁺-dependent protein phosphatase calcineurin, or as a calmodulin-like domain that is integrated into the sequence. The catalytic subunit of the ubiquitous isoforms of the protease calpain contains a carboxy-terminal domain that is very similar to calmodulin. However, the example of calpains is more complex and interesting, because their catalytic subunits also contain Ca²⁺-binding sites other than EF-hand motifs (8).

Several other EF-hand proteins have become well known, for example, troponin C as the Ca²⁺ receptor in myofibrils (9), recoverin as an 'actor' in the vision process, and calbindin, the expression of which is controlled by vitamin D. Recently, calbindin has been found to activate inositol monophosphatase, which is an important enzyme in the phosphoinositide signalling pathway (10). Finally, a group of EF-hand proteins, which are collectively called neuronal Ca²⁺ sensors, mediate neuronal functions such as the release of neurotransmitters and the activity of a family of K⁺ channels.

3- Ca²⁺-transporting proteins

Membrane-intrinsic proteins can create and shape Ca²⁺ signals by functioning as channels, ATPases (pumps), or exchangers that transport Ca²⁺ across membranes. Ca²⁺ channels mediate the penetration of Ca²⁺ into the cell and its exit from the sarco(endo)plasmic reticulum. In the plasma membrane, Ca²⁺ channels are gated by voltage changes, by ligand interactions, or by a mechanism that is linked to the emptying of intracellular Ca²⁺ stores and that has become known molecularly only recently (11). The voltage-gated channels have several sub-types (12), of which the L-type, which is the target of widely-used Ca²⁺ antagonists (13), is the best characterized. The most important ligand-operated channels are gated by neurotransmitters and, among them, the glutamate-operated channels are the most intensively studied (14). In the sarco(endo)plasmic reticulum, the opening of Ca²⁺ channels is regulated by Ca²⁺ itself — the process of Ca²⁺-induced Ca²⁺ release(15) — but this opening also requires ligands like inositol-(1,4,5)-trisphosphate (Ins(1,4,5)P₃) (16) or, in selected cell types, another endogenous ligand, cyclic ADP ribose (17), which releases Ca²⁺ through channels that are known as ryanodine receptors. Isoform 1 of this receptor (RYR1) is expressed predominantly in skeletal muscles, whereas isoform 2 (RYR2) is expressed in the heart and brain. The link between the activation of plasma-membrane receptors and the production of Ins(1,4,5)P₃ is well documented, but data on the link between plasma-membrane receptors and the production of cyclic ADP ribose are scarce. In addition, cyclic ADP ribose is thought to be produced extracellularly by an ectoenzyme (18) and therefore must be imported into cells to function. Interestingly, free ADP ribose — a product of NAD⁺ hydrolysis and a breakdown product of cyclic ADP ribose — that is produced inside cells has been shown to gate a Ca²⁺-entry channel, which is known as long transient receptor potential channel 2 (LTRPC2) (19). Finally, the nicotinic acid derivative of NADP⁺ — NAADP⁺ — is the latest addition to the family of Ca²⁺-mobilizing messengers (17)

Both the plasma membrane and the inner mitochondrial membrane contain Na⁺/Ca²⁺ exchangers (NCX and MNCX, respectively) that export Ca²⁺ from the cell (20,21) and the mitochondrial matrix (22), respectively. The high capacity plasma-membrane NCX is particularly active in excitable cells, which demand the periodic ejection of large Ca²⁺ loads. The less well-characterized mitochondrial exchanger (MNCX) is also particularly

active in excitable cells. Whereas the plasma-membrane NCX exchanges three Na^+ per one Ca^{2+} , and therefore responds both to the Na^+ and Ca^{2+} transmembrane gradients and to the voltage difference across the plasma membrane, MNCX seems to exchange two Na^+ per one Ca^{2+} . This electrically neutral operation of MNCX allows the export of Ca^{2+} from mitochondria in the presence of the large, negative membrane potentials that are maintained at the matrix side of the inner mitochondrial membrane by the respiratory chain (**Fig. 3**).

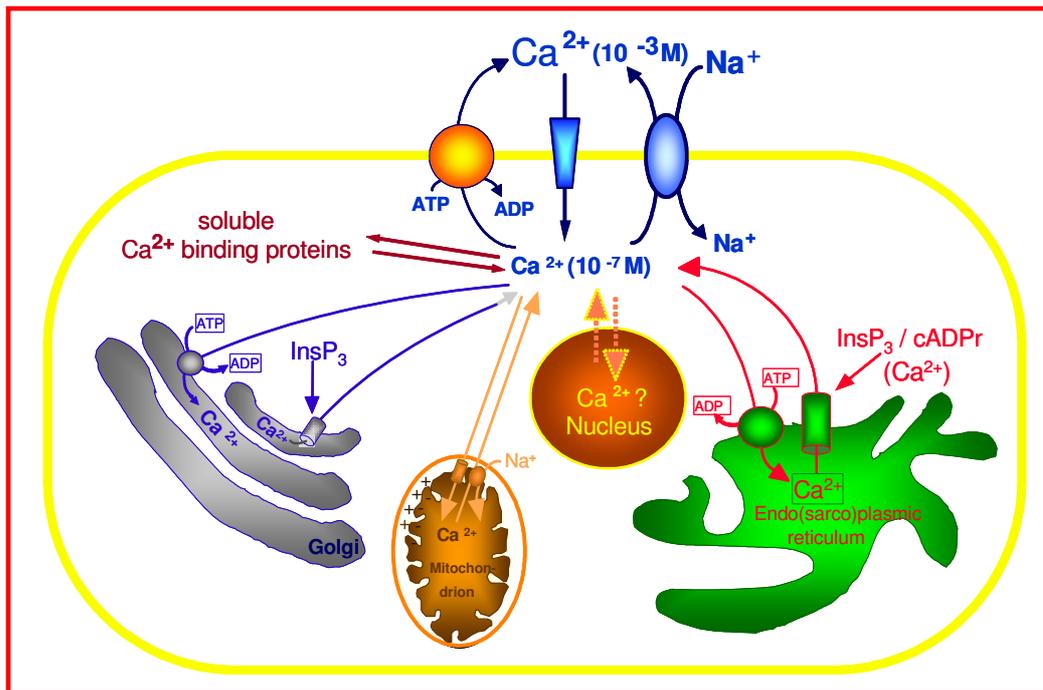


Figure 3: The Ca^{2+} transporters of cell membranes: PMCA: Plasma Membrane Ca^{2+} Pump; NCX: $\text{Na}^+/\text{Ca}^{2+}$ exchanger; SERCA Sarco/Endoplasmic Ca^{2+} Pump; InSP_3R : Inositol-1,4,5 Trisphosphate Receptor; RyR: Ryanodine Receptor

A spectacular advance in the field of membrane Ca^{2+} transporters was the determination of the tertiary structure of the sarco(endo)plasmic reticulum Ca^{2+} ATPase (the SERCA pump) by Toyoshima et al. (23,24). The structure was solved both in the Ca^{2+} -bound (E1) and Ca^{2+} -free (E2) conformations, which validated predictions of the membrane topology and molecular mechanism of the pump. The SERCA1 isoform is expressed predominantly in fast-twitch muscles, whereas SERCA2 is expressed in numerous non-muscle tissues as well. Splicing isoforms have also been described. One important aspect of the SERCA-pump function is the regulation by the membrane intrinsic protein phospholamban, the amino-terminal hydrophilic domain of which interacts with the active centre of the pump to keep it inactive (25). Phosphorylation of two neighbouring

residues in phospholamban detaches it from the pump and relieves the inhibition (**Fig. 4**)

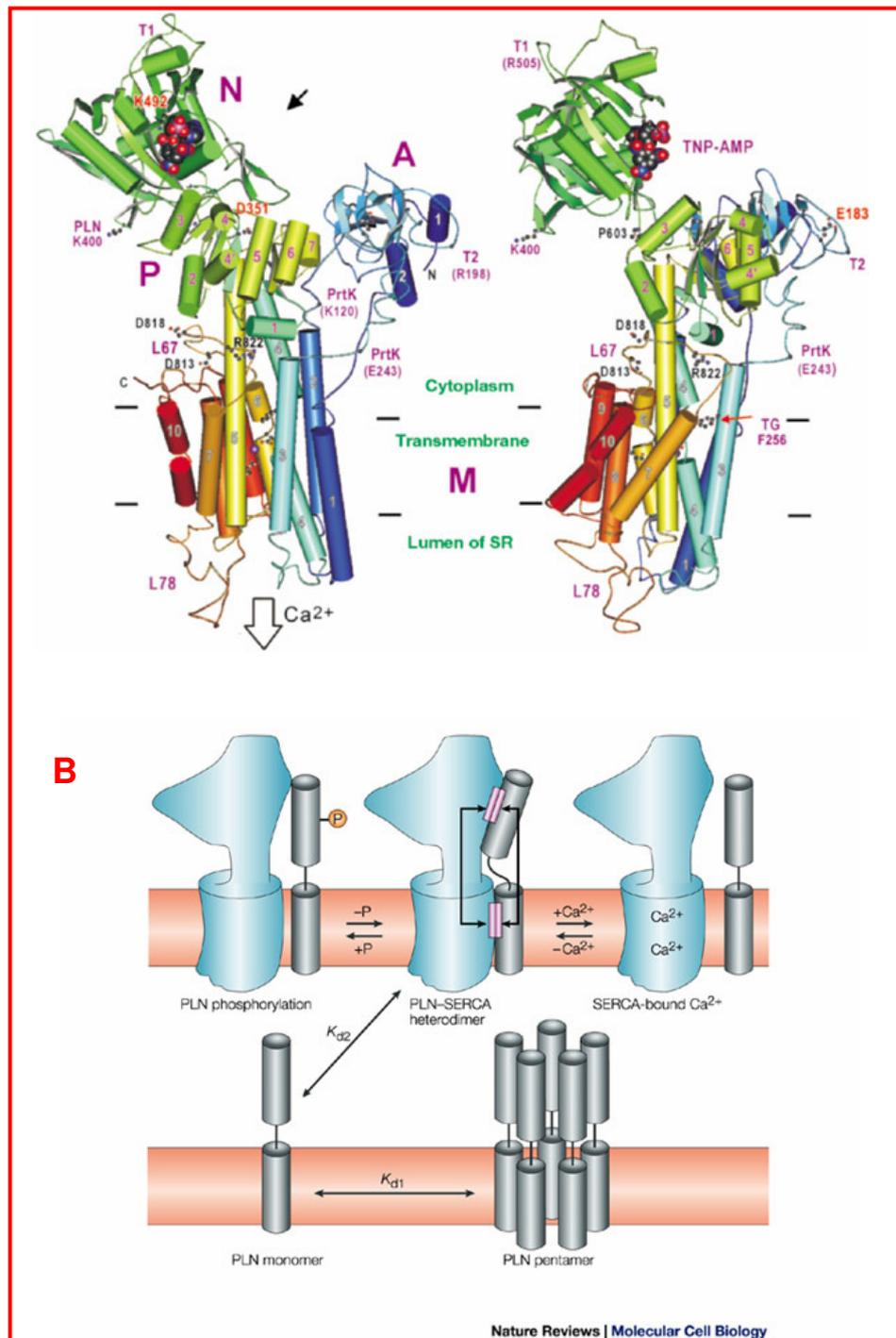


Figure 4: A) Architecture of the sarcoplasmic reticulum Ca²⁺-ATPase. Toyoshima, C. *et al.* Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. *Nature* 405, 647–655 (2000) **B) PLB/SERCA proposed interaction.** MacLennan D. and Kranias G. Phospholamban: a crucial regulator of cardiac contractility. *Nature Reviews Molecular Cell Biology* 4, 566-577 (2003)

Besides the well-known sarco/endoplasmic-reticulum Ca²⁺-transport ATPases (SERCA), animal cells contain a much less characterized P-type Ca²⁺-transport ATPase:

the PMR1/SPCA $\text{Ca}^{2+}/\text{Mn}^{2+}$ -transport ATPase. SPCA is targeted to the Golgi apparatus. Phylogenetic analysis indicates that it might be more closely related to a putative ancestral Ca^{2+} pump than SERCA. SPCA supplies the Golgi apparatus, and possibly other more distal compartments of the secretory pathway, with the Ca^{2+} and Mn^{2+} necessary for the production and processing of secretory proteins. In the lactating mammary gland, SPCA appears to be the primary pump responsible for supplementing the milk with high (60-100 mM) Ca^{2+} . It could also play a role in detoxification of cells overloaded with Mn^{2+} . Mutations in the human gene encoding the SPCA pump (ATP2C1) result in Hailey-Hailey disease (**Fig 5**), a keratinocyte disorder characterized by incomplete cell adhesion. Recent observations show that the Golgi apparatus can function as a Ca^{2+} store, which can be involved in setting up cytosolic Ca^{2+} oscillations. (26, 27)

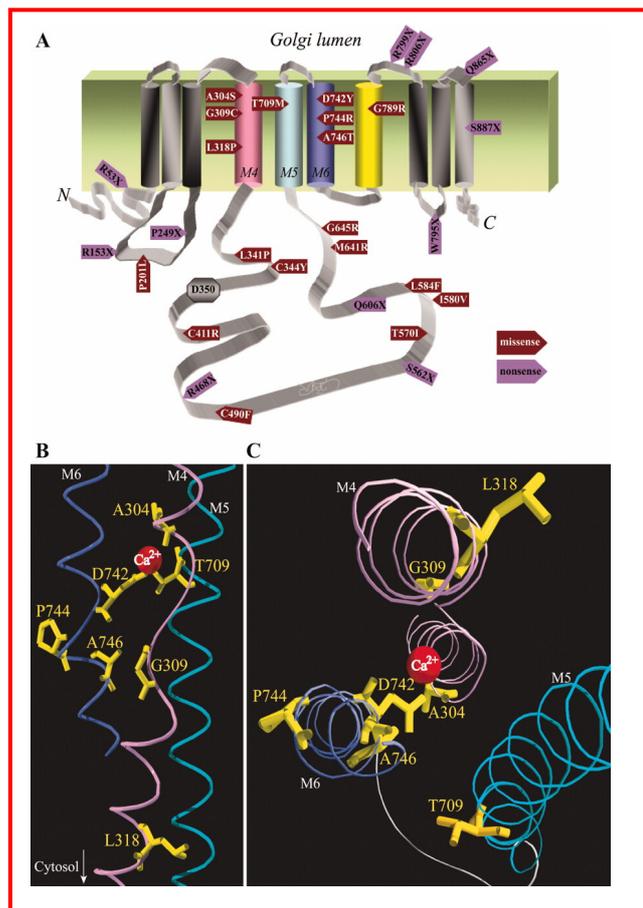


Figure 5: Hailey-Hailey disease (HDD) mutations in human Golgi/secretory pathway $\text{Ca}^{2+}, \text{Mn}^{2+}$ -ATPase (hSPCA1). Ton V K, Rao R. Functional expression of heterologous proteins in yeast: insights into Ca^{2+} signaling and Ca^{2+} -transporting ATPases. *Am J Physiol Cell Physiol.* 287(3):C580-9. (2004).

4- THE PLASMA MEMBRANE CALCIUM PUMP (PMCA)

The PMCA pump was discovered by H. J. Schatzmann forty years ago (28) as a system responsible for the active extrusion of Ca^{2+} from red cells. Later on, Schatzmann and Vincenzi (29) demonstrated that Ca^{2+} , when transported, activated the hydrolysis of ATP at the internal surface of the membrane. Following the initial observations on the red cell membrane a large number of studies on numerous cell types characterized the ATPase as a bona fide member of the family of transport ATPases that were later classified as P-type pumps (30,31). These pumps are characterized by the formation of an energized intermediate in which the γ -phosphate of ATP is transferred to an invariant Asp in the active site of the enzyme, resulting in the formation of a high-energy acyl-phosphate. As in all other P-type pumps, the reaction cycle of the calcium pumps oscillates between two conformational states: the E1 state, in which the enzymes have high affinity for Ca^{2+} , and the E2 state, in which the enzymes have much lower Ca^{2+} affinity. Ca^{2+} is bound to the cytosolic domain of the enzyme in the high-affinity conformation (E1), prior to the phosphorylation of the catalytic aspartic acid by ATP. A conformational change of the phosphorylated enzyme ($\text{E1P} \rightarrow \text{E2P}$) then exposes bound Ca^{2+} to the extracellular/luminal side of the membrane and promotes its release prior to the cleavage of the phosphorylated intermediate, after that, the pump returns to the E1 conformation (Fig. 6).

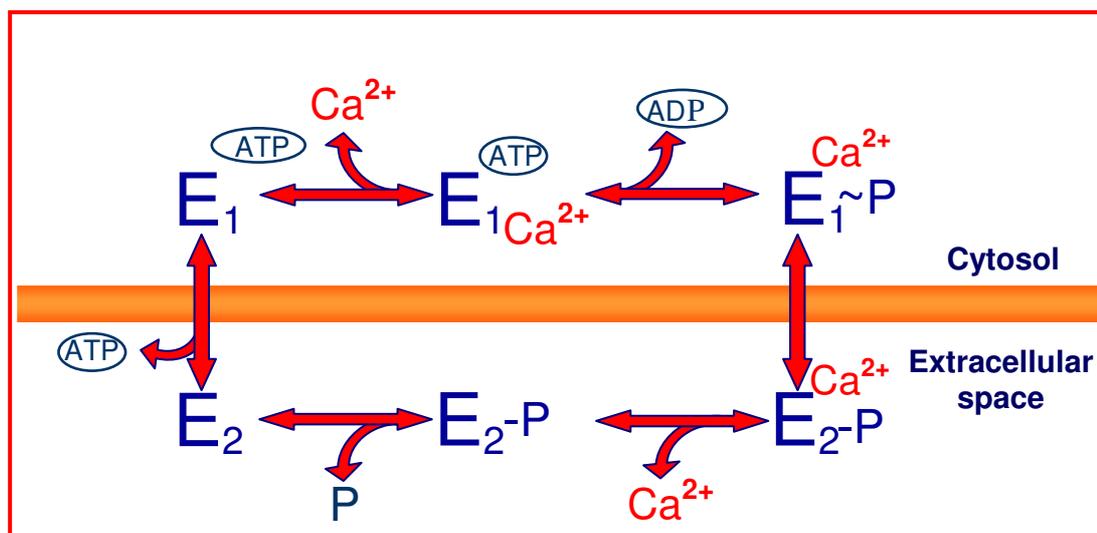


Figure 6: The P-type pumps catalytic cycle

Three P-type Ca^{2+} pumps are now recognized: The Golgi-membrane SPCA pump is the most recent entry in the field and has not yet been studied as extensively as the other pumps: thus, most of the discussion on general properties in the next paragraphs will concentrate on the pumps of the plasmamembrane (PMCA) and of the endo(sarco)plasmic reticulum (SERCA), for which considerable information is available. The two pumps share basic properties, but also have significant differences. For instance, the SERCA pump transports two Ca^{2+} ions per molecule of ATP hydrolyzed, whereas the Ca^{2+} /ATP stoichiometry of the PMCA pump is 1:1. Both pumps exchange Ca^{2+} for H^+ : early reports on the reconstituted PMCA pump had indicated an electroneutral $\text{Ca}^{2+}/\text{H}^+$ exchanger (**32**), but more recently work has instead convincingly shown partial electrogenicity, i.e. one H^+ is exchanged for one Ca^{2+} (**33**). The single Ca^{2+} binding site of the PMCA pump, which is thought to correspond to site II in the SERCA pump, is likely to be located on the cytosolic site of the membrane prior to the translocation step, and to face the exterior of the cell at its end. After releasing Ca^{2+} to the exterior of the cell, the pump becomes dephosphorylated and eventually returns to the E1 conformation. The process is fully reversible; the phosphorylated intermediate can be formed, under appropriate conditions, from inorganic phosphate as well.

As with all other P-type pumps, the PMCA pump is rapidly and irreversibly inhibited by micromolar concentrations of the phosphate analogue orthovanadate, which binds to the E2 conformer of the pump, thereby preventing the E2/E1 transition (**34**). The other general inhibitors of the P-type pumps are lanthanide ions, which have been shown to displace Ca^{2+} by combining with its high-affinity binding sites on the SERCA pump (**35, 36**). Since they allow phosphorylation to occur, they could replace Ca^{2+} as the metal ion that activates the phosphorylation as suggested for the SERCA pump (**36,37**). As expected, lanthanides also inhibit the PMCA pump, however, at variance with the SERCA pump (and other P-type pumps), they markedly increase the steady-state level of the phosphoenzyme (**38**). The effect is attributed to the inhibition of the hydrolysis of the phosphorylated intermediate by La^{3+} : it is very advantageous, as it permits the recognition of the PMCA pump in autoradiographic gels of membrane preparations containing much higher amounts of the SERCA or other P-type pumps.

The peptide caloxin 2A1 has been recently reported to inhibit the PMCA pump (**39**) by binding to putative extracellular domain 2 of isoform 1 (see below for information on the membrane topology of the pump) which is one of the two PMCA isoforms of

erythrocytes. Another component of the caloxin's peptide family, caloxin 1A1, apparently blocks the pump by perturbing its first extracellular domain. The caloxin findings suggest that transmembranes domains 1 and 2 play a role in the reaction cycle of the PMCA pump (40).

The PMCA pump is essential for the control of the cytosolic Ca^{2+} concentration in all eukaryotic nonmuscle cells, but in muscle cells, particularly heart cells, is likely to be of minor importance with respect to the much more abundant SERCA pump and to the high transport capacity sodium calcium exchanger of the plasma membrane. In eukaryotic cells, the amount of PMCA pump is thought not to exceed the level of 0.1% of the total membrane protein. This value may perhaps be underestimated in neurons, where the level of expression of the pump is apparently higher than in the other cells types (41).

4.1- ISOLATION AND PURIFICATION OF THE CALCIUM PUMP

The purification of the PMCA pump was first reported in 1979 by Niggli et al. (42) using calmodulin affinity chromatography. The decision to use calmodulin column was prompted by previous work on erythrocyte membranes that had shown that calmodulin activated the pump (43, 44). Red blood cell membranes were solubilized with Triton X-100 and stabilized with phosphatidylserine and the preparation was applied to the column. Most of the membrane proteins, including the Mg^{2+} -dependent ATPase activity, were eliminated with Ca^{2+} containing buffers. EDTA then eluted from the column the Ca^{2+} dependent ATPase as a major band of about 125 KDa accompanied by a minor band of about 205 KDa. Both bands were phosphorylated by ATP in the presence of Ca^{2+} and Mg^{2+} : the heavier band was later shown to be a dimer of ATPase. The PMCA was then successfully reconstituted as an active pump in liposomes. A crucial advance that resulted from the work on liposomes was the detailed analysis of the role of acidic phospholipids as activators. In the first purification experiments the pump had already been maximally active in the absence of calmodulin. This was so because the calmodulin column had been saturated with the acidic phospholipid phosphatidylserine, which was later found to activate the pump alternatively to calmodulin (see next section). Replacement of phosphatidylserine with the zwitterionic phospholipid phosphatidylcholine soon produced purified preparations that were fully responsive to calmodulin. Another important result of the early work on the purified

PMCA was the precise definition of the pattern of proteolysis, especially of that by calpain (45). It occurred in two steps: the first calpain cleavage removed about half of the calmodulin binding domain and the portion of the pump downstream of it. A second cut then truncated the pump at the beginning of the calmodulin-binding domain. The fully active 124 kDa peptide resulting from the two calpain cuts could be easily purified since it was not retained by calmodulin columns, and was later used to demonstrate that the calmodulin binding sequence acted as an autoinhibitory domain. It bound to two sites in the cytosolic domain of the pump close to the active centre (see next section), maintaining the pump inactive (46, 47). The irreversible activation of the pump by calpain was thus evidently due to the removal of the autoinhibitory region, i.e., the calmodulin-binding domain. It may have significance in pathological states of the cell, when the resting level of free Ca^{2+} remains elevated for long periods, thus activating calpain (48).

4.2- CLONING OF THE PUMP AND RECOGNITION OF ISOFORMS

The primary structure of the pump was deduced in 1988 using rat brain (49) and human teratoma (50) cDNA libraries, respectively. Two rat sequences contained 1176 (129.5 KDa) and 1198 (132.6KDa) amino acids, respectively, the human sequence 1220 residues (137.7KDa) corresponding to that of the latter rat isoform. All regions of functional importance present in other ion-transporting ATPases were soon identified. The translated sequence had the calmodulin-binding in the carboxyl terminal cytosolic tail. It also had a domain near the amino terminus very rich in basic aminoacids which was soon found to have a role in the interaction with activatory acidic phospholipids (see below).

It soon emerged that several PMCA isoforms were encoded by a multigene family, and that additional isoform variability was produced by alternative RNA splicing of each gene transcript.

All regions important to the catalytic function of P-type pumps were found to be highly conserved in the PMCA sequences and, as expected, no isoform diversity was detected in these regions, which are essential to the catalytic function of the pump. The structural motifs responsible for the membrane-folding pattern of the P-type pumps also tend to be conserved, i.e., isoform diversity was not found to involve the general arrangement of the transmembrane domains of the pump.

4.3- THE PLASMA MEMBRANE Ca^{2+} PUMPS: STRUCTURAL AND REGULATORY CHARACTERISTICS.

The cloning work predicted the PMCAs to contain 10 membrane-spanning segments (TM), with the NH_2 and COOH termini located on the cytosolic side of the membrane. The bulk of the protein mass faces the cytosol and consists of three principal domains: the intracellular loop between TM 2 and 3, a large unit between TM domains 4 and 5, and the extended tail following the 10th transmembrane domain. The first intracellular loop between membrane spanning domains 2 and 3 corresponds to the transduction or actuator domain of the SERCA pump (**51**), and is thought to play a role in the long range transmission of conformational changes occurring during the transport cycle. The large cytosolic region (about 400 residues) between transmembrane segments 4 and 5 contains the catalytic domain (the ATP binding site and the invariant aspartate that forms the acyl phosphate intermediate). Finally, the extended COOH -terminal tail is the major regulatory domain of the PMCAs. On the basis of sequence comparison and computer modeling (unpublished), the overall 3-D structure of the PMCAs resembles that of the SERCA pump. However, differences between the two types of calcium pumps are also evident: the COOH -terminal tail is much shorter in the SERCA than in the PMCA pumps, and the PMCAs contain a basic sequence (see above) in the transduction domain that binds regulatory acidic phospholipids (**52**).

Long before the first PMCA cDNA was cloned, it was realized that the pump was tightly regulated by Ca^{2+} bound to the modulator protein calmodulin (see above), which decreased the K_d of the pump for Ca^{2+} from 10- 20 μM in the resting state to less than 1 μM : calmodulin interacts with the C-terminal tail of the pump (**53**). In the absence of calmodulin, and as long as the Ca^{2+} concentration in the vicinity of the pumps is low (< 50–100 nM), the PMCAs remain in an auto-inhibited state. This is so because the calmodulin binding domain in the C-terminal tail makes intramolecular contacts with the first and second cytosolic loop of the pump, thus presumably preventing substrate access to the active site (**46, 47**). Ca^{2+} loaded calmodulin de-inhibits the pump by removing its C-terminal tail from the intramolecular binding sites, increasing Ca^{2+} affinity and raising V_{max} . (**54**). This regulatory mechanism is similar to that of other Ca^{2+} -calmodulin dependent enzymes. In the case of one of these enzymes, calmodulin dependent protein kinase I (CaMKI), the crystal structure has convincingly shown that in the absence of Ca^{2+} -calmodulin the COOH -terminal regulatory domain (which

overlaps with the calmodulin binding domain.) interacts with multiple sites in the catalytic core, preventing substrate access and obstructing ATP binding (55) exactly as predicted for the case of the PMCA pump. One additional comment is in order on the interplay between the PMCA pump and calmodulin: unique among calmodulin targets, the pump can be activated by the C-terminal half of calmodulin. Since the latter has peculiarly high Ca^{2+} affinity, it is probably Ca^{2+} -saturated even at the low level of Ca^{2+} in the resting cell. It could thus interact with the N-terminal half of the calmodulin binding domain, maintaining the pump in a low state of activity even under conditions of resting cytosolic Ca^{2+} (56).

In addition to calmodulin, kinase mediated phosphorylations also activate the pump. The effect was first identified in the pump of heart sarcolemma membranes and attributed to the cAMP protein kinase (PKA) (Fig 7). The phosphorylation step increased both the V_{\max} and the Ca^{2+} affinity of the pump, decreasing the K_m (Ca^{2+}) to about $1 \mu\text{M}$ in the absence of calmodulin. The consensus site for PKA (KRNSS) was identified downstream of the calmodulin-binding domain in the C-terminal region of one of the isoforms of the pump (PMCA1) (57).

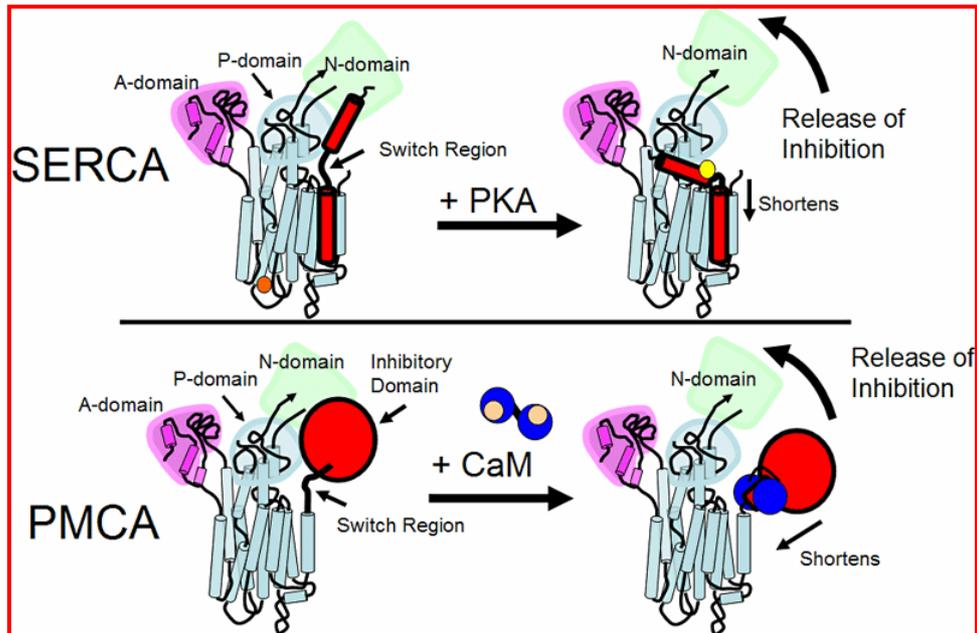


Figure 7: Release of Ca-ATPase Inhibition. Motional restriction of catalytically important motions of the nucleotide binding domain (N) of the Ca-ATPase occurs through the binding of either PLB (A) or an autoinhibitory domain (shown in red), which underlies the regulation of calcium transport in the sarcoplasmic reticulum or plasma membrane of the heart. In both cases, the stabilization of helical structure within a conformational switch region results in a shortening of the physical dimensions and the necessary release of the inhibitory interactions with the N-domain.

The problem of activation by protein kinase C (PKC) is more complex. Stimulation of the pump by this kinase has been described by several authors (58-64), also in the solubilized, purified erythrocyte Ca^{2+} pump (65). The phosphorylation occurs in the C-terminal region, involving both serine and threonine residues, one of the phosphorylation sites being a threonine residue within the calmodulin binding domain. Based on peptide data (66), phosphorylation would be expected to prevent binding of calmodulin to the pump while causing a calmodulin-like activation (67).

As mentioned above, PMCA pump stimulation is also induced by acidic phospholipids and polyunsaturated fatty acids. The observation that acidic phospholipids are more effective than calmodulin, i.e. that they decrease the K_m (Ca^{2+}) to values lower than those induced by calmodulin (68), has led to the suggestion that acidic phospholipids and calmodulin activate the Ca^{2+} ATPase by separate mechanisms involving different binding sites. Work with distinct proteolytic fragments of the PMCA pump and with synthetic peptides has indeed revealed two separate phospholipid binding regions in the pump: one corresponding to the calmodulin binding domain, which has basic character (69), and the other, as mentioned, situated in the first cytosolic loop (52). The suggestion that a substantial portion of the phospholipid stimulation of the pump was apparently mediated by the calmodulin binding domain is in line with the observation that also other calmodulin-regulated proteins, i.e., neuromodulin and myosin light-chain kinase, are activated by acidic phospholipids.

The phospholipid effect could be important *in vivo*, as the ATPase in the membrane is presumably surrounded by an amount of phosphatidylserine adequate for about 50% of maximal activation (70). However, the concentration of phosphatidylserine, and of the other abundant acidic phospholipids in the plasma membrane, is presumably invariant. By contrast, the concentration of phosphatidylinositol (PI) and of its two phosphorylated products (PIP1 and PIP2) in the membrane is modulated in response to first messenger challenges to the cell, making them more attractive as potential modulators. Thus, an activation/deactivation cycle of the pump linked to the metabolism of PI and designed to modulate the export of Ca^{2+} from the cell as demanded by stimuli that promote intracellular Ca^{2+} release, appears possible (71).

In addition to mediating the regulation by Ca^{2+} -calmodulin, the C-terminal region of the PMCA has also been shown to be affected by the irreversible activator calpain (see above 45, 72, 73), and to mediate the interaction with PDZ domain proteins. The interaction may be important for the local organization of Ca^{2+} signaling domains at the

plasma membrane and/or for anchoring Ca^{2+} regulatory complexes to the cytoskeleton. The PMCA s are also activated by self-association (74). Even if self-association still permits to bind Ca^{2+} -calmodulin (75), in the oligomerized state there is no further activation by calmodulin. The functional significance of the dimerization (oligomerization) of the PMCA s, which is mediated by the calmodulin binding domain (76), is a controversial point. An increase of Ca^{2+} -ATPase activity at high enzyme concentrations has been attributed to pump oligomerization (75). However, more recent findings (77), suggest that the dimerization does not correlate with the function of the pump, or has minimal effects on it (78).

4.4- ISOFORMS OF THE PMCA PUMP

As mentioned, four basic PMCA isoforms are encoded by four independent genes named ATP2B1 –ATP2B4, that map respectively to the following human chromosomal loci: 12q21-q23, 3p25-p26, Xq28 and 1q25-q32 (79-82).

The size of the four human genes ranges from about 60kb (PMCA3) to over 350 kb (PMCA2) (83-86), however, the number and size of protein-coding exons are highly conserved among the 4 genes, suggesting a common origin by duplication of an ancestor gene. In all genes the first exon encodes a 5' prime untranslated sequence that precedes the ATG starting codon in the second exon. The transcribed proteins have a homology of about 70-80 % and maintain the general folding features. Residues differ among isoforms especially in the N- terminal protrusion and the C-terminal tail.

PMCA1 and PMCA4 are ubiquitously distributed in animal cells and are presumed to carry out housekeeping functions to maintain Ca^{2+} homeostasis. PMCA1 appears to be ubiquitously expressed since the earliest states of development, as highlighted in studies of mice pump phenotypes. PMCA4 is widely expressed in human tissues and supports PMCA1 in maintaining Ca^{2+} homeostasis. In human cells PMCA4 is particularly abundant in kidney, erythrocytes and testis, where it is the principal isoform. PMCA2 and 3 are tissue restricted, and found in particular in the nervous system, suggesting functional specificity.

In the nervous system PMCA2 and PMCA3 are present with regional and developmental distribution differences. PMCA2 is abundant in the Purkinje cells of cerebellum and in the outer hair cells of the inner ear (87, 88), while PMCA3 is abundant in the habenula and in the choroid plexuses (89, 90). However, traces of

PMCA3 are also present in skeletal muscle (91). Hippocampus cells, instead, are particularly rich in PMCA1, suggesting an additional tissue specific function of this ubiquitous isoform. Outside the nervous system, PMCA2 is also abundantly expressed in lactating mammary glands, and significant levels of this isoform have also been found in the kidney. During development, isoforms 2 and 3 appear at about day 12.5 of gestation and vary in levels and time distribution: PMCA2 starts to be detected in high levels in the dorsal root ganglia and in the retinoblasts of the developing eye. PMCA3 could be important for the correct development of the organs, as it is widely expressed in tissues from day 12.5 up to day 16.5 of gestation, when it start to be selectively expressed in cell types (92).

Information on the differential functional properties of the 4 basic PMCA isoforms is scarce. The calmodulin affinity of the two tissue-specific variants, PMCA2 and PMCA3, is much higher than that of the two ubiquitously expressed isoforms PMCA 1 and PMCA 4 (41), and the sensitivity to calpain is particularly elevated in PMCA1 (93). When tested in the native intracellular environment the two recombinant ubiquitous isoforms are far less effective in controlling temporary Ca^{2+} increases induced by the challenging of model cells overexpressing the pumps with agonists that produce Ca^{2+} transients, than the two tissue specific isoform PMCA2 and PMCA3 (94).

4.5- SPLICING VARIANTS

RNAs encoding for plasma membrane calcium pumps are subjected to alternative splicing (95) at two different sites, considerably increasing the number of pumps variants. About twenty of the many putative variants have been identified, some, however, only at the RNA level. The physiological rationale for the existence of such a great number of tissue specific variants is one of the open problems in the PMCA pump area.

Supplementary exons are alternatively transcribed in the proximity of the two main regulatory domains of PMCAs. Splice site A is located upstream of the phospholipid binding domain in the first intracellular loop of the pump, and splice site C is placed inside the calmodulin binding domain in the C terminal cytosolic tail of the protein. A third splice site, Splicing site B, is now considered an aberrant splice variant. Alternative splices at sites A and C have been extensively characterized for human and rat isoforms and reviewed in numerous papers (96-97). A possible additional variant has

so far only been identified in amphibians (98). The scheme of **Fig. 8** summarizes the variants so far identified for each isoform.

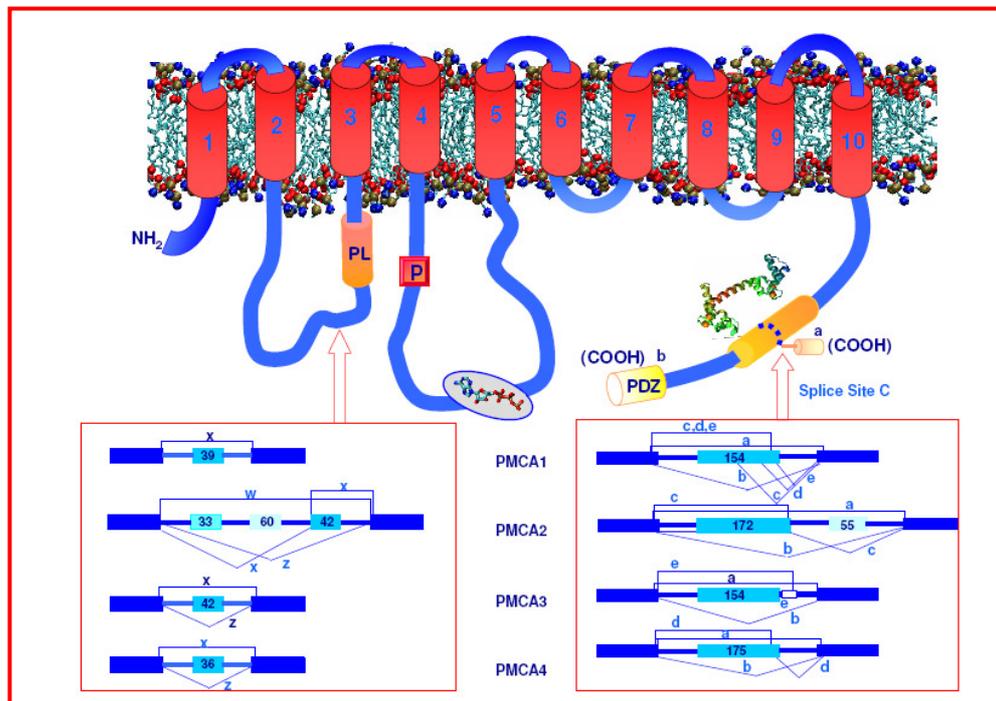


Figure 8: Membrane Topology domains and splicing variants of the PMCA pump

Two different nomenclature systems (**TABLE 1**) have been developed to identify the splicing variants (99, 100). According to the most commonly used one, insertions in splice A are addressed with letters from *z* to *w*, according to the increased number of inserted exons: (for instance, in variant *w* of PMCA2 three exons are included, in variant *x* only one exon of 42 bp is transcribed, in variant *y* two exons, one of 33 and the other of 60bp are inserted, in variant *z* all supplementary exons are excluded). At splice site C the alternatively transcribed exons are identified by letters from *a* to *e*. At variance with site A, at splice site C alternative exons are fully or partially transcribed determining changes of the reading frame and the premature truncation of the protein.

	<i>Original Nomenclature</i>	<i>Alternative Nomenclature</i>
Splice site A	z	AI
	x	AII
	y	AIV
	w	AIII
	v	AV
Splice site B	a	CII
	b	CI
	c	CIII
	d	CIV
	e	CV
	f	CIV

PMCA1 is characterized by the presence in all identified mature transcripts of the sequence of an exon of 40 bp, which is only alternatively included in the other isoforms, therefore all known PMCA 1s correspond to the *x* variant. PMCA 2 presents a more complex pattern of possible splice variants for site A. Up to three different exons (respectively of 33, 60 and 42 bp) can be transcribed. So far only 4 of the 8 possible combinations have been identified. Variant *y* has been so far detected in rat but not in humans. Variant *v*, in which a fourth transcribed exon is inserted at splice site A, has been found only in bullfrog. PMCA 3 and 4 exist only as variants *x* or *z*, including or excluding an exon of 42 or 36 nucleotides, respectively.

Splicing at site C occurs with different degrees of complexity for each isoform, however common options can be identified. A multitude of variants is generated by exon-internal splice sites, read-through into adjoining introns, and inclusion or omission of entire

exons. As mentioned, such a combination of events usually leads to reading frame changes with the consequent insertion of premature stop codons, and modification of the C terminal structure of the pump. The most common modification determined by total or partial insertion of exons is the truncation of the calmodulin binding domain, with changes in the regulatory mechanism of the pumps. Unmodified (no inserts) C-terminal proteins are designated as *b* variants. In general, for PMCA1 and PMCA 4 a single exon is involved in alternative splices at site C, while PMCA2 and PMCA3 include or exclude partially or completely 2 exons.

The tissue distribution and the targeting to plasma membrane domains, of the splice isoforms has not been determined in detail, but it is known that the ubiquitous variants PMCA1 *xb* and PMCA4 *b* localize predominantly in the basolateral part of the plasma membranes of polarized cells. PMCA 2 *wb* is expressed in mammary gland cells and localizes instead in the apical section of the cell membrane. The *w* insert probably has a general role in the membrane sorting of PMCA variants: in polarized cell lines the splicing *w* is a determinant for the targeting of the pumps to the apical plasma membrane (101). Interestingly, PMCA 2 *wa* is selectively expressed in cochlear and vestibular stereocilia, as shown by immunoprecipitation from purified hair bundles of rat utricles (102). Recently, it has also been demonstrated that the combination of splice *w* at site A and *a* at site C is essential for the correct targeting of the protein to the stereocilia (103).

4.6- PROTEIN INTERACTORS OF PMCA PUMPS

Naturally, the established function of PMCAs is to expel Ca^{2+} from cytosol. But an additional role for the pump has been recently suggested, on the basis of newly discovered interactions of the pumps with molecules involved in signaling pathways (104, 105): the pump would function as a modulator of signal transduction. For example, in cardiomyocytes, where the Na/Ca^{2+} exchanger is far more important than the PMCA pump in the extrusion of Ca^{2+} , PMCA4 has been shown to form a ternary complex with $\alpha 1$ -syntropin and nitric oxide synthase1 (NOS-1). The physical interaction with NOS-1 induces a negative regulation of the NO production, and the overexpression of $\alpha 1$ -syntropin synergistically increases the inhibitory effect. NOS-1 binds to the PDZ binding domain in the C-terminal tail of PMCA, while the $\alpha 1$ -

syntrophin interacts with the distal region of the large intracellular loop of the protein (residues 652-840) **(106)**.

The same region of the main intracellular loop of PMCA4 binds the tumor suppressor Ras-associated Factor 1 (RASSF1), a Ras effector protein involved in H-Ras-mediated apoptosis. Co-expression of RASSF1 and PMCA4b inhibits the epidermal-growth-factor dependent activation of Ras **(107)**.

Another interactor of the pump that binds to its second intracellular loop (residues 501-575) is calcineurin, whose modulation of the transcriptional activity of NFAT is inhibited by the interaction. PMCA would therefore be an upstream effector of calcineurin/NFAT signaling, acting as a regulator of differentiation and adaptation of different tissues **(108)**. It would seem logical to think that the interaction of the pump with partners should be isoform-specific, and the search of isoform-specific interactors has recently been successful. The 14-3-3 protein has been found to interact with PMCA4 but not with PMCA2, when coexpressed in model cells. The interaction occurs at the N-terminal portion of the pump and is inhibitory **(109)**.

Interactors of the PMCA pumps were also identified using the C-terminal tail of the pump, which contains a PDZ binding sequence, as bait in yeast double screening experiments. These include members of the membrane associated guanylate-kinase family (MAGUK), cytoskeletal proteins, the Na⁺/H⁺ exchanger regulatory factor 2 (NHERF2), the Ca²⁺/Calmodulin- dependent serine protein kinase (CASK) and the novel protein PMCA-interacting single-PDZ protein (PISP). CASK co-precipitates with PMCA4 from brain and kidney tissues lysates. The CASK protein is a co-activator of promoters containing transcription of T-elements. Overexpression of PMCA downregulates T-element dependent reporter activity **(105)**.

As for PISP, it transiently interacts with PMCA *b* variants and plays a role in the sorting of the pump to or from the plasma membrane **(110)**.

PMCA4b interacts with the PDZ domains of several MAGUKs, a result that has led to the suggestion that C-terminal splicing would generate pump isoforms that may be differentially recruited to multifunctional protein complexes involved in Ca²⁺ regulation **(111)**.

PMCA2b and PMCA4b have been used to explore the association with NHERF1 and NHERF2. The interaction with the latter was found to be specific for PMCA2b, i.e., it did not occur with PMCA4b. PMCA2b apparently preferred NHERF2 over NHERF1 as an interactor. The interaction may provide a link between the Ca²⁺ pump and the actin

cytoskeletal network, potentially stabilizing the pump in a particular membrane microdomain, possibly allowing its regulation by co-assembled protein kinase A. Alternatively, NHERF2 mediated co-clustering of multiple PMCA 2*b* molecules may facilitate their oligomerization, which has been shown in a previous section to lead to calmodulin-independent pump activation (112).

One more example of an attractive partner protein for *b* variants of all PCAs via their PDZ binding domain is the Ania 3/Homer protein. The Homer family of proteins has gained attention as a component of the post-synaptic density and is involved in the coupling of NMDA glutamate receptors to the emptying of Ca^{2+} stores. Coexpressed Ania 3 colocalized with PMCA at the plasma membrane of polarized MDCK epithelial cells, and endogenous Ania3/Homer and PMCA2 co-expressed in the soma and dendrites of primary hippocampal neurons (113). **Fig. 9** offers a comprehensive view of the partners of PMCA pumps identified so far and of their approximate sites of interaction.

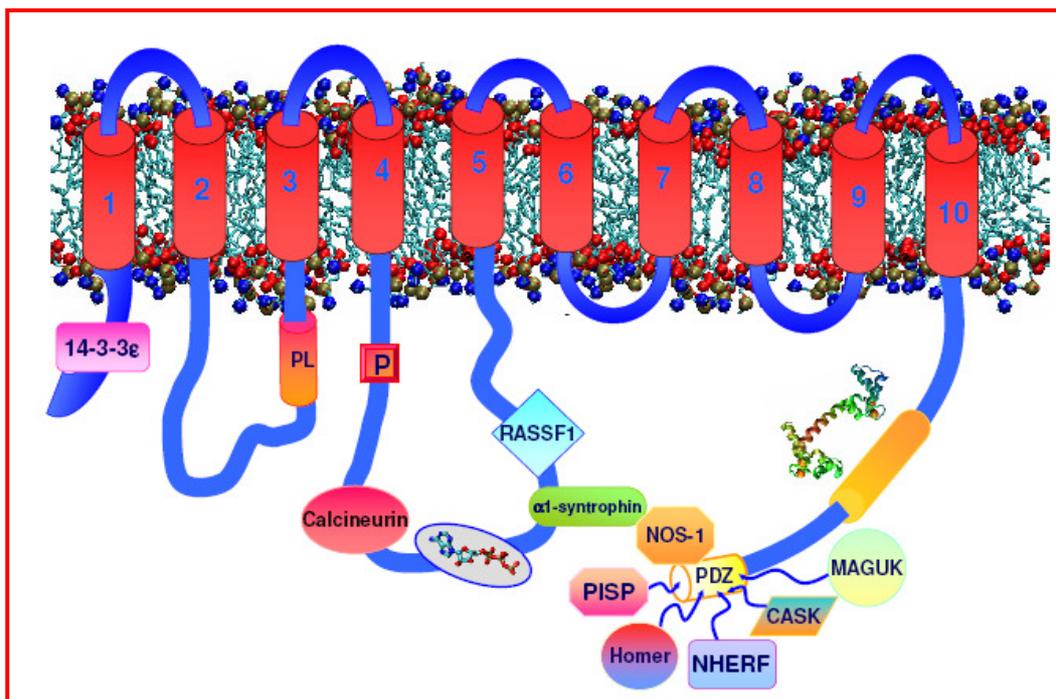


Figure 9: Interactors of Plasma Membrane Ca^{2+} ATPase pumps

4.7- PMCA PUMP AND DISEASE

4.7.1- PMCA PUMP KNOCKOUT MICE

Knock out mice have been developed and the phenotypes extensively studied for all isoforms of the PMCA pumps with the exception of PMCA 3. As this isoform is widely expressed in tissues of developing embryos its role may be critical for normal development of gestation. Hopefully, PMCA 3 gene ablation studies will be soon forthcoming.

PMCA1 was disrupted by targeting the catalytic phosphorylation site, but homozygous knockouts resulted in embryonic lethality. Null mutant embryos were identified up to day 3 of gestation, but not during the period of organogenesis. The inability to generate adult live animals underlines the important role of this housekeeping isoform from the earliest ages of development. On the other hand, heterozygous mutants did not to present a pathological phenotype, even if the smooth muscle of blood vessels (the portal vein was examined) appeared apoptotic. As this smooth muscle does not express the other ubiquitous isoform PMCA 4, the absence of PMCA 1 gene on one allele was evidently inefficiently compensated (**114**).

Mice subjected to the targeted ablation of the PMCA 4 survived and appeared outwardly healthy. Histological analysis of organs provided no evidence of major tissue alterations or *in vivo* cell death (**115**). Thus, in spite of its ubiquitous expression, PMCA 4 appears to be less critical than PMCA 1 in the maintenance Ca^{2+} homeostasis. A major phenotypic alteration was nonetheless detected, and this was male infertility. Sperm was unable to achieve efficient hyperactivated motility, and was unable to reach and fertilize the egg. This was evidently due to the fact that isoform 4 represents 90% of all PMCA pumps expressed in testis cells and underlies the importance of PMCA in the regulation of Ca^{2+} homeostasis in sperm cells, which evidently depend on it for an appropriate motility (**116**).

PMCA 4 was also found to be crucial in modulating calcium signals in B lymphocytes. Triggering of the B cell receptor (BCR) in null mutant mice caused an enhanced transient increase in cytosolic Ca^{2+} , which was attenuated by CD22 (a B cell restricted transmembrane glycoprotein) via stimulation of Ca^{2+} efflux. The observation suggests a direct interaction between PMCA 4, CD22 and SHP-1 tyrosine phosphatase (**117**).

The analysis of the phenotypes of PMCA 2 knockout mice has revealed interesting characteristics. Though the animals appeared fairly normal at birth, they started to

present balance impairment around day 10 **(118)** Recording of the auditory brain response revealed that they were deaf and the analysis of the vestibular inner ear showed the absence of otoconia. It was also observed that sensory hair cells started to degenerate after day 10. The most severely affected animals also presented partial loss of nerve cells. Unexpectedly, Purkinje neurons, even if they abundantly express PMCA 2, were unaltered, and the cerebella maintained normal organization and cell distribution. Thus, the balance defect in knockout mice was not due to a general (cerebellar) neuronal impairment but it was rather determined by the absence of otoconia that sense gravity and equilibrium changes in the vestibular system.

Similar phenotypes were observed in mice presenting naturally occurring mutations of the PMCA 2 gene. The first historically identified animals with mutated sequence of the pump were the deafwaddler mice **(88)**. The deafwaddler mutation will be described in more detail in the next section, but is mentioned here because one of the deafwaddler mutations generates a null mutant. Indeed, deafwaddlers carry either a point mutation (G283S) in the first intracellular loop of the PMCA 2 protein (dfwj) or a frame shift mutation at codon 471 generating a null mutant (dfw2j, dfw3j). Another point mutation of the PMCA 2 gene (K412E) was identified by a later study in the deaf Wriggle Sagami mouse. The aminoacidic substitution in the fourth transmembrane domain determined an altered membrane targeting of the pumps that was therefore no longer expressed in the stereocilia **(119)**.

The PMCA 2 knockout mouse (and the PMCA 2 mutations) thus shows that this isoform has a crucial role in the auditory system. Interestingly, in the outer hair cells PMCA 2 is specifically and selectively expressed in the stereocilia. PMCA 1 and PMCA 4 are also present in these sensory cells, but are confined to the basolateral section of the plasma membrane.

4.7.2- HEREDITARY DEAFNESS AND OTHER DISEASE CONDITIONS.

Studies aimed at characterizing disease features caused by genetic defects of the PMCA proteins, as well as by variations of gene transcription levels have now begun to appear **(120)**, but for a long time no human disease was directly linked to a defect in any of the genes of the four different isoforms of the PMCA pump. Only very recently two mutations in the gene encoding for PMCA 2 have been identified in humans with hearing impairment. Schultz et al. have described a hypofunctional variant of *ATP2B2*

that introduced a V586M substitution in the vicinity of the active site of the protein and enhanced the auditory defect in patients with an accompanying homozygous mutation in the gene of Cadherin 23 (121). Another mutation in the PMCA 2 gene, leading to a G293S substitution, has been observed by our group in a child with hearing impairment in an Italian family (122). It is of interest that the G293S mutation involves the same mutation only 10 residues downstream the site of the deafwaddler mutation. These findings, together with the results of the studies on knockout mice for the *ATP2b2* gene described in the preceding section, highlight the essential role of PMCA2 in mammalian hearing. Apparently, no other PMCA isoform can adequately compensate for the loss of PMCA 2, or of PMCA 2 activity, in sensory hair cells emphasizing the special properties of PMCA2, and of its splice variant present in the stereocilia (see below). The PMCA 2 gene mutations identified so far as causative of hereditary deafness are summarized in **Fig. 10**. A particularly interesting mutation has recently been found in a mouse with hereditary hearing in loss. The phenotype of the mouse and the biochemistry of the mutated pump are presently being analyzed and the results obtained so far will be described below. The special interest of the mutation lies in the fact that it affects a transmembrane domain of the pump: Transmembrane domain substitutions are normally assumed to alter the membrane targeting of the PMCA pumps, yet, the mutated pump is correctly delivered to the plasma membrane.

In most cell types non-genetic alterations of PMCA activity contribute to a variety of pathological states. In turn, modifications of expression levels or abnormal proteolysis of PMCA have been observed in different organs and systems. In neuronal differentiation, for instance, up-regulation of calcium channels to trigger calcium dependent events is associated with PMCA up regulation, to respond to the increased demand for calcium extrusion (123). In particular, PMCA 2 and 3 isoforms, specifically expressed in the central nervous system, are up regulated during the development of granule neurons of cerebellum (124). Isoforms PMCA 2 and PMCA 3 are normally up-regulated in neuronal development, as shown in the neuroblastoma cell line IMR-32 (125). PMCA 4 is less involved in neuronal differentiation, and may even be down-regulated (126), whereas PMCA1 is a determinant for neurite extension as shown in PC-6 neuronal cells (127), where knocking down of the PMCA 1 gene by siRNA leads to the loss of nerve growth factor-mediated neurite elongation (128).

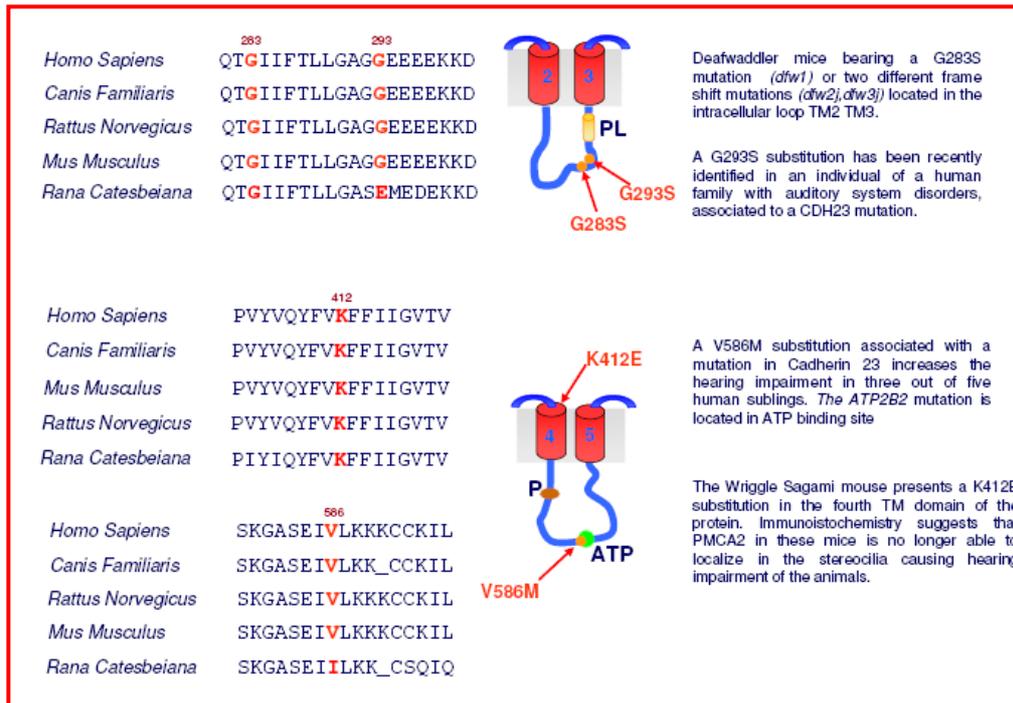


Figure 10: Deafness-associated mutations of Plasma Membrane Ca²⁺ ATPase

Alterations of calcium homeostasis play a particularly important role in brain aging, brain damage by ischemia/anoxia, and several neurodegenerative diseases. Free radicals affect membrane Ca²⁺ transport by acting directly on the PMCA or by deranging the lipidic bilayer that hosts it. For instance, in Alzheimer patients' neurons, calcium homeostasis is altered. The amyloid beta-peptide (Abeta), which accumulates in AD brain, is neurotoxic: its addition to cultured hippocampal neurons caused altered calcium homeostasis preceded by the impairment of Na-K ATPase and Ca ATPase activities (129).

Cardiac hypertrophy and myopathy may also be associated with abnormalities in calcium homeostasis linked to modifications of the PMCA pump. Sarcolemmal membranes from rats with pressure and hypertension-related cardiac hypertrophy presented increased activity of the PMCA. This is likely to be an adaptive reaction to facilitate the removal of Ca²⁺ from myocardial cells during the development of hypertrophy (130). In atherosclerosis and restenosis the regulation of apoptosis is controlled by the transcription factor C-myb, whose binding to the PMCA 1 gene promoter represses pump transcription in vascular smooth cells muscle at G1/S cell cycle interface (131).

Pancreatic islet and beta cells of pancreas contain a unique combination of PMCA isoforms, predominantly PMCA 1*b*, 2*b*, 3*a*, 3*c* and 4*a*. Evidently, functionally distinct PMCA isoforms participate in Ca²⁺ homeostasis in insulin secreting cells (**132,133**). The PMCA activity of pancreatic islets is suppressed by high glucose, and is therefore altered in diabetes (**134**).

In kidney, the expression of PMCA isoforms can be selectively modulated by pathophysiological stimuli. At variance with the constant expression level of PMCA1 and PMCA4 isoforms, the expression of PMCA 2 and PMCA 3 in tubules has been shown to be depressed in hypercalciuric rats (induced by feeding with a low phosphate diet) in comparison with hypocalciuric rats. In brain and liver, however, PMCA 2 and PMCA3 were unchanged, suggesting a specific role for these isoforms in tubular Ca²⁺ reabsorption (**135, 136**). Familial hypercalcemia has been associated with global defects of PMCA function (**137**).

Finally, PMCA 2 mRNA has been detected in higher level in some breast cancer cell lines, where it may be overexpressed by a factor of up to 100 fold.

Experimental procedures

1- Baculovirus Expression System

Baculoviruses are the most prominent viruses known to affect the insect population. They are double-stranded, circular, supercoiled DNA molecules in a rod-shaped capsid. (*Summers, M. and Anderson, D. (1972) J. Virol. 9, 710.*) Two of the most common isolates used in foreign gene expression are *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and *Bombyx mori* (silkworm) nuclear polyhedrosis virus (BmNPV). Wild-type baculoviruses exhibit both lytic and occluded life cycles that develop independently throughout the three phases of virus replication. The following are characteristics of the three phases:

1. **Early Phase:** In this phase (also known as the virus synthesis phase), the virus prepares the infected cell for viral DNA replication. Steps include attachment, penetration, uncoating, early viral gene expression, and shut off of host gene expression. Actual initial viral synthesis occurs 0.5 to 6 h after infection.
2. **Late Phase:** In this phase (also known as the viral structural phase), late genes that code for replication of viral DNA and assembly of virus are expressed. Between 6 and 12 h after infection, the cell starts to produce extracellular virus (EV), also called non-occluded virus (NOV) or budded virus (BV). The EV contains the plasma membrane envelope and glycoprotein (gp) 64 necessary for virus entry by endocytosis. Peak release of extracellular virus occurs 18 to 36 h after infection.
3. **Very Late Phase:** In this phase (also known as the viral occlusion protein phase), the polyhedrin and p10 genes are expressed, occluded virus (OV)—also called occlusion bodies (OB) or polyhedral inclusion bodies (PIBs)—are formed, and cell lysis begins. Between 24 and 96 h after infection, the cell starts to produce OV, which contains nuclear membrane envelopes and the viral polypeptides gp41 and gp74. Multiple virions are produced and surrounded by a crystalline polyhedra matrix. The virus particles produced in the nucleus are embedded within the polyhedrin gene product and a carbohydrate-rich calyx.

1.1- Baculoviruses as Expression Vectors

The major difference between the naturally occurring in vivo infection and the recombinant in vitro infection is that the naturally occurring polyhedrin gene within the wild-type baculovirus genome is replaced with a recombinant gene or cDNA. These genes are commonly under the control of polyhedrin and p10 promoters. In the late phase of infection, the virions are assembled and budded recombinant virions are released. However, during the very late phase of infection, the inserted heterologous genes are placed under the transcriptional control of the strong AcNPV polyhedron promoter. Thus, recombinant product is expressed in place of the naturally occurring polyhedrin protein. Usually, the recombinant proteins are processed, modified, and targeted to the appropriate cellular locations.

1.2- Cytopathogenesis

As the recombinant infection advances, several morphological changes take place within the cells. The timing of the infection cycle and the changes in cell morphology vary with the insect cell line and strain of baculovirus used. The metabolic condition of the culture and growth medium used also can affect the timing of baculovirus infection. The following morphological changes are typical of monolayer *Spodoptera frugiperda* (Sf9) cells infected with recombinant AcNPV.

1. **Early Phase:** Infection begins with the adsorptive endocytosis of one or more competent virions by a cell in a high metabolic state (peak replication rate). The nucleocapsids pass through the cytoplasm to the nucleus. When the virions enter the nucleus, they release the contents of the capsid. Within 30 min of infection, viral RNA is detectable. Within the first 6 h of infection, the cellular structure changes, normal cellular functions decline precipitously, and early-phase proteins become evident.

2. **Late Phase:** Within 6 to 24 h after infection, an infected cell ceases many normal functions, stops dividing, and is logarithmically increasing production of viral genome and budded virus. The virogenic stroma (an electron dense nuclear structure) becomes well developed. Infected cells increase in diameter and have enlarged nuclei. The cells may demonstrate reduced refractivity under phase contrast microscopy. Infected cultures stop growing.

3. **Very Late Phase:** Within 20 to 36 h after infection, cells cease production of budded virus and begin the assembly, production, and expression of recombinant gene product.

In monolayer cultures, areas of infection display decreased density as cells die and lyse. Likewise, in suspension cultures, cell densities begin to decrease. Infected cells continue to be increased in diameter and have enlarged nuclei. The cytoplasm may contain vacuoles, and the nuclei may demonstrate granularity.

1.2- Generating a Recombinant Virus by Homologous Recombination

Using homologous recombination to generate a recombinant baculovirus is outlined in **Fig. 2a**. The most common baculovirus used for gene expression is AcMNPV. AcMNPV has a large (130-kb), circular, double-stranded DNA genome. The gene of interest is cloned into a transfer vector containing a baculovirus promoter flanked by baculovirus DNA derived from a nonessential locus—in this case, the polyhedrin gene. The gene of interest is inserted into the genome of the parent virus (such as AcMNPV) by homologous recombination after transfection into insect cells.

Typically, 0.1% to 1% of the resulting progeny are recombinant. The recombinants are identified by altered plaque morphology. For a vector with the polyhedrin promoter, as in this example, the cells in which the nuclei do not contain occluded virus contain recombinant DNA. Detection of the desired occlusion-minus plaque phenotype against the background of greater than 99% wild-type parental viruses is difficult. A higher percentage of recombinant progeny virus (nearly 30% higher) results when the parent virus is linearized at one or more unique sites located near the target site for insertion of the foreign gene into the baculovirus genome.(*Kitts, P.A., Ayres, M.D. and Possee, R.D. (1990) Nucleic Acids Res. 18, 5667., Hartig, P.C. and Cardon, M.C. (1992) J. Virol. Methods 38, 61*) To obtain an even higher proportion of recombinants (80% or more), linearized viral DNA that is missing an essential portion of the baculovirus genome downstream from the polyhedrin gene can be used.(*Kitts, P.A. and Possee, R.D. (1993) Biotechniques 14, 810.*) These approaches can take more than a month to purify plaques, amplify the virus, and confirm the desired recombinants.

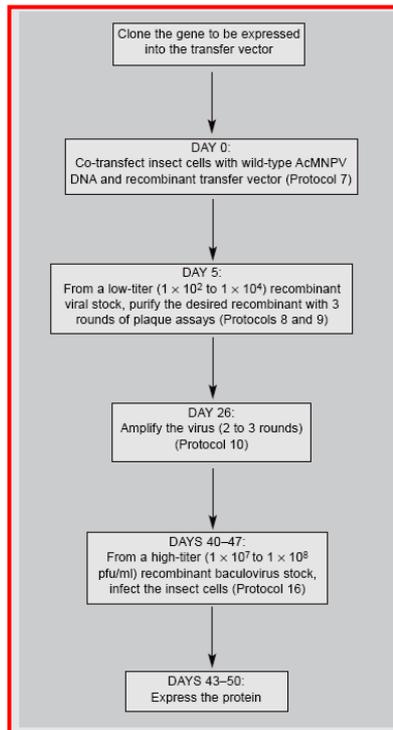


Figure 2a: Generating a recombinant baculovirus by homologous recombination

1.4-Generating a Recombinant Virus by Site- Specific Transposition

A faster approach for generating a recombinant baculovirus (*Luckow, V.A., Lee, S.C., Barry, G.F., and Olins, P.O. (1993) J. Virol. 67, 4566.*) uses site-specific transposition with Tn7 to insert foreign genes into bacmid DNA propagated in *E. coli*. The gene of interest is cloned into a pFASTBAC vector, and the recombinant plasmid is transformed into DH10BAC competent cells which contain the bacmid with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the pFASTBAC plasmid can transpose to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by antibiotic selection and blue/white screening, since the transposition results in disruption of the *lacZ α* gene. High molecular weight mini-prep DNA is prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA is then used to transfect insect cells. The steps to generate a recombinant baculovirus by site-specific transposition using the BAC-TO-BAC Baculovirus Expression System are outlined in **Fig. 2b**.

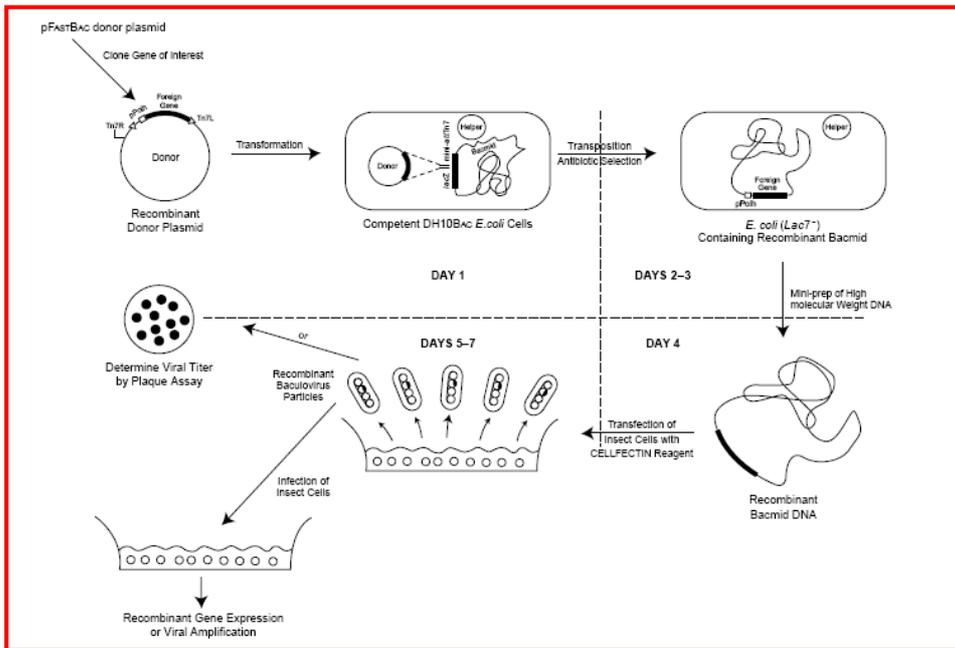


Figure 2b: Generation of recombinant baculoviruses and gene expression with the BAC-TO-BAC expression system

2- Cloning and virus generation of PMCA2 z/b and w/a variants

2.1- Construction of the recombinant baculoviruses

The HindIII fragment from pcDNA3-PMCA2 zb and the Not/KpnI fragment from pmRFP-PMCA2 wa were inserted in pFASTBAC1, non-fusion vector (no fusion tags are present in the vector) and pFASTBAC HtB fusion vector (fusion with the N-terminal 6xHis tag and the TEV protease cleavage site). Recombinant baculoviruses were produced in Sf9 insect cells by the BAC-TO-BAC™ system (Gibco-BRL, Life Technology Inc., Basel, Switzerland).

2.2- Plasmid DNA isolation from bacteria. Miniprep

E. Coli cells carrying the plasmid of interest were incubated overnight at 37°C at constant shaking (200-220 rpm) in 5 ml of Luria-Bertani (LB) broth (10g/L Bacto Tryptone, 5g/L Bacto yeast extract, 10g/L NaCl) supplemented with the appropriate antibiotic (ampicillin 75 µg/µL or kanamycin 40 µg/µL). The cells were harvested by centrifugation at 13000 rpm (microcentrifuge Biofuge, Haeraeus) for 1 min and the plasmid DNA was isolated using the “NucleoSpin Plasmid” protocol (Macherey-Nagel).

The protocol is based on SDS/alkaline lysis of the *E. Coli* cells followed by neutralization of the resulting lysate, binding of plasmid DNA to silica membranes columns and elution under low ionic strength conditions of pure plasmid DNA.

2.3- Plasmid DNA isolation from bacteria. MaxiPrep

E. Coli cells carrying the plasmid of interest were incubated overnight at 37°C in 300 ml of LB medium supplemented with antibiotics as described previously. The cells were harvested by centrifugation at 5000 rpm in a Beckman Coulter centrifuge (rotor JA10) for 15 min at 4°C. Plasmid DNA was extracted using Large Scale Plasmid MaxiPrep (Qiagen). The procedure employs an SDS/alkaline method to prepare cleared lysate, followed by neutralization of the lysate and plasmid DNA binding to anion exchange resins. The purified plasmid DNA was eluted from the column and concentrated by an alcohol precipitation. The concentration and quality of the purified DNA was measured with a UV spectrophotometer at OD₂₆₀₋₂₈₀

2.4-DNA digestion and agarose gel electrophoresis

Digestion of DNA was performed with restriction endonucleases which recognize unique DNA palindromic sequences at which they hydrolyze the phosphoester linkage.

An analytical restriction enzyme reaction contained:

- 1 µg DNA (in TE 1X buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8)
- 2 µl 10x buffer (depending on the restriction enzyme)
- 1 µl restriction enzyme (10 units/µl activity)
- Sterile H₂O up to a final volume of 20 µl

The reaction mix was incubated for 1 hour on a water bath at 37°C.

A preparative restriction enzyme reaction contained:

- 3-10 µg DNA (in TE 1X)
- 10 µl 10X buffer mix
- 3 µl restriction enzyme (10 units/µl activity)
- Sterile H₂O up to a final volume of 100 µl

The reaction mix was incubated for 2 hours on a water bath at 37°C.

The DNA samples were loaded on 1% agarose (Type I Low EEO, Sigma) gels together with 1x loading buffer (0.04% bromophenol blue, 0.04% xylene cyanol, 5% glycerol in H₂O) and ran at 80 V for approximately 30 min.

The size of the separated DNA strands was determined by comparison of their relative position to that of the DNA strands of the 1000bp DNA Ladder (MBI Fermentas).

2.5- DNA fragments purification from agarose gels

Following agarose gel electrophoresis, the gel slice containing the desired DNA band was excised with a sharp scalpel and purified using the “NucleoSpin Extract” kit (Macherey-Nagel). The protocol is based on DNA binding to a silica membrane in the presence of chaotropic salts and elution of pure DNA under low ionic strength conditions with TE 1x buffer. Contaminations like salts and soluble macromolecular components are prevented by a washing step with an ethanolic buffer.

2.6- DNA ligation

DNA ligation was performed by incubating the DNA fragments with the appropriately linearized cloning vector in the presence of 2-4 units of T4 DNA ligase (3 units/ μ L), 10x T4 ligase buffer (50 mM TrisHCl pH 7.5, 10 mM MgCl₂, 10 mM DTT, 25 μ g/ml BSA, 1 mM ATP) and sterile water to a final volume of 10 μ L . A vector: insert molar ratio of 1:3 was usually used.

The reaction mix was incubated overnight at 16°C

2.7- Transformation of competent *E. Coli* cells

TOP10 chemically competent cells (Invitrogen) which have been kept on freezer storage were thawed on ice. 1-200 ng of plasmid DNA were added to the competent cells and the transformation mix was kept on ice for 30 min. The cells were heat-shocked for 30-40 seconds at 42° and then incubated for 30 min at 37°C in 500 μ l of LB broth. The mix was plated out on LB agar which contained the antibiotic ampicillin that selects for transformants.

2.8- DNA sequencing

DNA sequencing was performed by the BMR Genomics Center of the University of Padova.

2.9- Generation of the Recombinant Bacmid

The baculovirus shuttle vector (bacmid), bMON14272 (136 kb), present in DH10Bac *E. coli* (Invitrogen) contains:

- A low-copy number mini-F replicon
- Kanamycin resistance marker
- A segment of DNA encoding the LacZ α peptide from a pUC-based cloning vector into which the attachment site for the bacterial transposon, Tn7 (miniattTn7) has been inserted. Insertion of the mini-attTn7 does not disrupt the reading frame of the LacZ α peptide.

The bacmid propagates in *E. coli* DH10Bac as a large plasmid that confers resistance to kanamycin and can complement a *lacZ* deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate such as Blue-gal or X-gal and the inducer, IPTG.

Recombinant bacmids (composite bacmids) are generated by transposing a mini- Tn7 element from a pFastBac donor plasmid to the mini-attTn7 attachment site on the bacmid. The Tn7 transposition functions are provided by a helper plasmid

2.10- Transformation of the DH10Bac *E. coli* cells

For transformation of MAX Efficiency DH10Bac chemically competent cells with the pFastBac construct, positive controls for transposition were included (*i.e.* pFastBac expression plasmid).

1. Thaw on ice one vial of MAX Efficiency® DH10Bac™ competent cells for each transformation.
2. For each transformation, 100 μ l of the DH10Bac cells were gently mixed and transferred into a pre-chilled, 15 ml round-bottom polypropylene tube.
3. Appropriate amount of plasmid DNA was added into the DH10Bac cells.
 - pFastBac construct: 1 ng (5 μ l)
 - pFastBac™ control plasmid: 1 ng
 - pUC19 control: 50 pg (5 μ l)
4. The cells were incubated on ice for 30 minutes and were subjected to heat shock for 45 seconds at 42°C without shaking
5. The tubes were transferred into the ice and chilled for 2 minutes

6. 900 µl of S.O.C. Medium (Bacto-tryptone, 20 g/L, Bacto-yeast extract, 5 g/L, NaCl, 0.5g/L, 1M KCl, 2.5 ml/L) was added at room temperature.

7. **For pFastBac™ transformations:** tubes were shaken at 37°C at 225 rpm for 4 hours.

For pUC19 transformation: at 37°C at 225 rpm for 1 hour.

For each pFastBac™ transformation: 10-fold serial dilutions of the cells were prepared (10^{-1} , 10^{-2} , 10^{-3}) with S.O.C. Medium. Plated 100 µl of each dilution on an LB agar plate containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, and 40 µg/ml IPTG.

For the pUC19 transformation: the cells were diluted 1:100 with S. O.C. Medium. Plated 100 µl of the dilution on an LB agar plate containing 100 µg/ml ampicillin.

8. Plates were incubated for 48 hours at 37°C. White colonies were picked for analysis (Insertions of the mini-Tn7 into the mini-*att*Tn7 attachment site on the bacmid disrupt the expression of the LacZ α peptide, so colonies containing the recombinant bacmid are white in a background of blue colonies that harbor the unaltered bacmid.) (**Fig. 2c**)

9. White colonies were picked and restreaked on fresh LB agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, and 40 µg/ml IPTG. The plates were incubated overnight at 37°C. From a single colony was confirmed to have a white phenotype on restreaked plates containing Bluo-gal and IPTG, were inoculated the liquid culture containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline.

10. Recombinant bacmid DNA was isolated using the Marligen MiniPrep Kit (Catalog no. 11449-022)

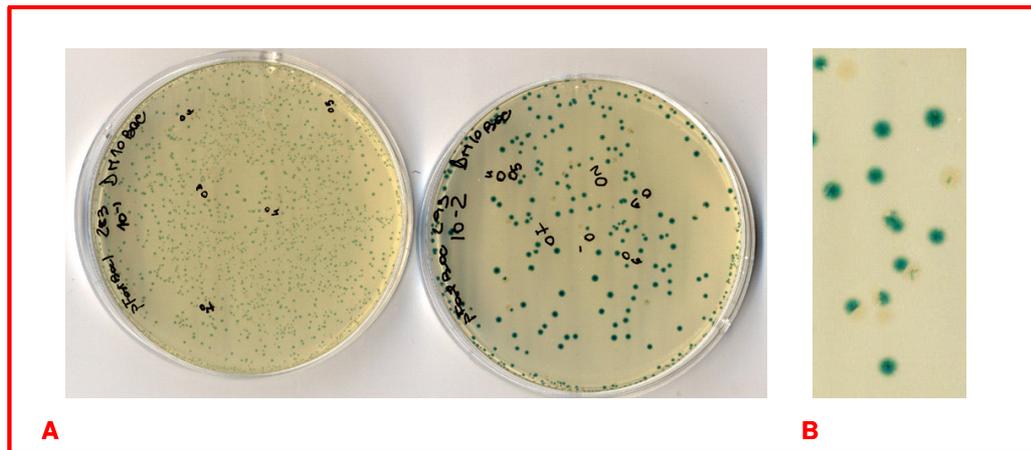


Figure 2c: Recombinant Bacmids colonies selected with X-gal: **A.** Different diluted dishes with recombinant bacmid colonies, the negative colonies are blue; **B.** A zoom in of a dish where is possible distinguish blue colonies from white recombinant colonies

2.11- Analysis of Recombinat bacmid DNA by PCR

The bacmid contains M13 Forward (-40) and M13 Reverse priming sites flanking the mini-*att*Tn7 site within the *lacZα*-complementation region to facilitate PCR analysis. (Fig. 2d)

Primers sequences:

M13 Forward (-40) 5'd [GTTTTCCAGTCACGAC]3'

M13 Reverse 5'd [CAGGAAACAGCTATGAC]3'

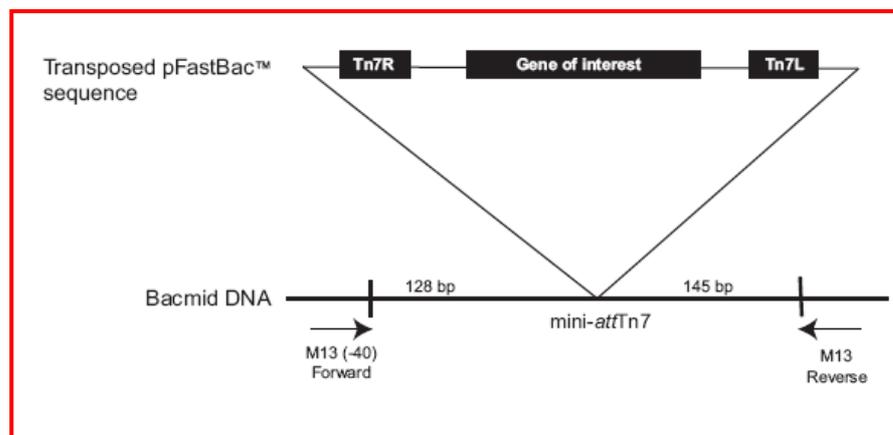


Figure 2d: PCR analysis of Recombinant bacmid DNA

For each sample, was set up the following 50 μ l PCR reaction in a 0.5 ml microcentrifuge tube:

- Recombinant bacmid DNA (100 ng) 1 μ l
- 10X PCR Buffer (appropriate for enzyme) 5 μ l
- 10 mM dNTP Mix 1 μ l
- 50 mM MgCl₂ 1.5 μ l
- PCR Primers (1.25 μ l each 10 μ M stock) 2.5 μ l
- Sterile Water 38.5 μ l
- Platinum *Taq* polymerase (5 units/ μ l) 0.5 μ l
- Total Volume 50 μ l

The PCR was amplified using the following cycling parameters:

- Initial Denaturation : 3 minutes 93°C 1X
- Denaturation: 45 seconds 94°C
- Annealing: 45 seconds 55°C 25-35X
- Extension: 5 minutes 72°C
- Final Extension: 7 minutes 72°C 1X

5-10 μ l from the reaction was removed and analyzed by agarose gel electrophoresis.

2.12- Transfection of Insect cells

The Bacmid transfection was performed using Cellfectin reagent (Invitrogen). This reagent is a 1:1.5 (M/M) liposome formulation of the cationic lipid N,N₁, N₂, N₃, N₄-Tetramethyl-N, N₁, N₂, N₃-tetrapalmitylspermine (TM-TPS) and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water.

The transfection was assayed as followed:

1. 9×10^5 per well insect cells was seeded in a 6-well or 35 mm tissue culture plate with 2 ml of growth medium containing antibiotics (*e.g.* 2 ml of Sf-900 II SFM medium (GIBCO) containing 1 μ g/ml gentamicin final concentration).
2. The cells were allowed to attach at 27°C for at least 1 hour.
3. **For each transfection sample** was prepared bacmid DNA:Cellfectin Reagent complexes as follows in 12 x 75 mm sterile tubes.
 - a. 1 μ g of purified bacmid DNA was diluted in 100 μ l of unsupplemented Grace's Medium (GIBCO).
 - b. Cellfectin Reagent was mixed thoroughly before use by inverting the tube 5-10 times. 6 μ l of reagent was removed and diluted in 100 μ l of unsupplemented Grace's Medium.

- c. The diluted bacmid DNA with the diluted Cellfectin Reagent were combined (total volume was ~210 μ l) and incubated for 15 to 45 minutes at room temperature.
4. While DNA: lipid complexes were incubated, the media from the cells was removed and washed once with 2 ml of unsupplemented Grace's Medium.
5. 0.8 ml of unsupplemented Grace's Medium was added to each tube containing the DNA:lipid complexes DNA:lipid complexes were mixed gently and added the to each well containing cells.
6. The cells were incubated in a 27°C incubator for 5 hours.
7. The DNA:lipid complexes were removed and added 2 ml of complete growth media (Sf-900 II SFM containing antibiotics) to the cells.
8. The cells were incubated in a 27°C humidified incubator for 5 days or 7 days.
- . At 5th day post transfection, **V_o virus** was produced and isolated from medium.

2.13- Virus amplification

A volume of 100 ml of Sf9 insect cells, with a density of 2×10^6 cells/ml, were infected with the viral particles, using a multiplicity of infection (MOI) of 0.1 to 1. MOI is defined as the number of virus particles per cell. The MOI was calculated using the following formula:

$$\text{Inoculum required (ml)} = \frac{\text{MOI (pfu/cell)} \times \text{number of cells}}{\text{titer of viral stock (pfu/ml)}}$$

After 48 hour incubation, cells were sedimented at 1300 rpm for 10 minutes at 4°C. The supernatant, containing the virus, was filtered to eliminate Sf9 cell residues and stored at 4°C.

3- Expression and purification of PMCA2 *zb* and *wa* variants

3.1 - Infection and expression of PMCA2 in insect cells (Figure 2e)

To express PMCA 2 isoforms a suspension cell culture of Sf9 insect cell line was used. The cell growth was carried out at 27°C in Sf-900 II SFM medium with L-Glutamine (GIBCO), 10% (v/v) Foetal Bovine Serum (GIBCO) 1 μ g/ml Gentamicin (GIBCO).

When the cells in the medium reached the mid-logarithmic phase of growth, corresponding to a density of 1×10^6 to 2×10^6 cells/ml, cells were ready to be infected with the virus.

Infection was performed using a multiplicity of infection (MOI) of 1 to 5.

A time course was performed to determine the expression kinetics for the protein, considering also the incidence of the degradation process as long as the expression proceeds.

The expression was finally carried out at 27°C by infecting 1 litre of culture. Cells were harvested 18 hour after the infection by centrifugation at 1500 rpm with a JA14 Rotor in an Avanti J-20 XP Centrifuge (Beckman Coulter) for 10 min at 4°C .

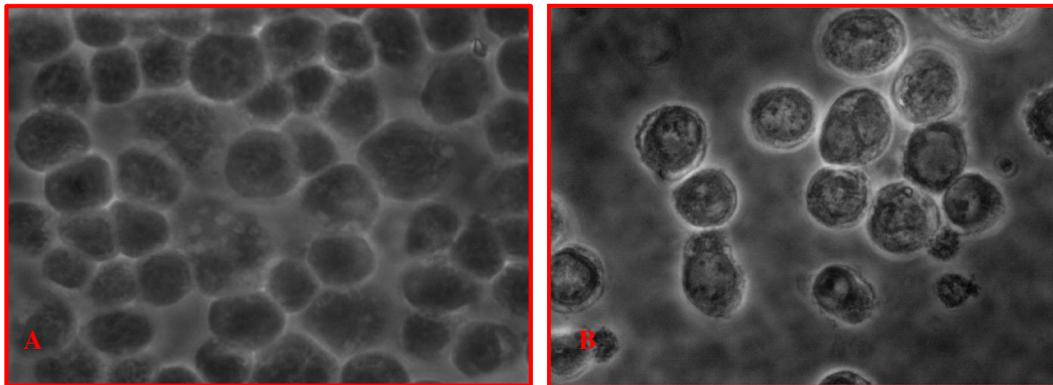


Figure 2e : Morphological differences between uninfected and infected sf9 cells
A. Uninfected Sf9 cells continue to divide and form a confluent monolayer **B.** Sf9 cells infected with recombinant baculovirus, these cells stop dividing and enlarge

3.2- Sf9 cell membrane preparation

Sf9 cells from a litre of culture were washed three times with 25 mM Tris pH 7.5, 0.15 M NaCl, 0.1 mM PMSF, centrifuging each time at 1500 rpm with a JA14 Rotor (Avanti J-20 XP Centrifuge, Beckman Coulter) for 10 min at 4°C .

The pellet was resuspended in 20 ml of 10 mM Tris pH 7.5, 1 mM DTT and Protease Inhibitors (Cocktail Tablets complete EDTA-free (Roche)).

The resuspended pellet was then homogenised with an Ultra Turrax T25 Basic (IKA Labortechnik) to lyse the cells. To the homogenised material Sucrose to 10% (w/v) and KCl 150 mM final concentration were added.

The lysed cells were centrifuged at 750g for 10 min at 4°C with a centrifuge Z383K (Hemle). To the supernatant EDTA to a 10 mM final concentration was added. The supernatant was finally centrifuged at 120000Xg for 45 min at 4°C.

The pellet was resuspended in 4 mM Tris-HCl pH 7.5, 10% (w/v) Sucrose and Protease Inhibitors.

3.3- Solubilisation of PMCA2 proteins

Sf9 isolated membranes were treated with an amount of detergent C₁₂E₈ equal to the total protein amount within the membranes.

Membranes were incubated with the detergent at 4°C for 1 hour in rotation.

Solubilised material was then centrifuged at 120000Xg for 30 min at 4°C.

To the supernatant were added 0.5 mg/ml Phosphatidylcholine, 15% (v/v) Glycerol, 3 mM DTT, 50 µM CaCl₂.

3.4- Calmodulin affinity chromatography

Membrane proteins extract was incubated at 4°C for 1 hour rotating with a Calmoduline Sepharose 4B (Amersham Biosciences) opportune bed volume, previously equilibrated with 20 mM Hepes

pH 7.2, 120 mM KCl, 1 mM MgCl₂, 100 µM CaCl₂, 0.32% (w/v) C₁₂E₈, 0.5 mg/ml Phosphatidylcholine, 15% (v/v) Glycerol, 3 mM DTT.

The resin with the bound protein is then harvested by centrifugation at 2000Xg in a centrifuge Z383K (Hemle) for 3 min at 4°C, and the supernatant (*flow through*) is conserved.

The resin was washed with the equilibration buffer and the protein was finally eluted by incubation at 4°C for 30 min rotating in 20 mM Hepes pH 7.2, 120 mM KCl, 1 mM MgCl₂, 0.05% (w/v) C₁₂E₈, 0.5 mg/ml Phosphatidylcholine, 15% (v/v) Glycerol, 10 mM EDTA, 3 mM DTT.

The resin was centrifuged at 2000 Xg for 3 min at 4°C and to the supernatant, containing PMCA2zb were added 1.5 mM MgCl₂ and 4.8 µM CaCl₂.

The protein concentration of the samples from the membrane preparation and the protein purification was determined by using the Lowry method.

3.5- His Tag affinity purification

PMCA 2 isoforms were subjected to His GraviTrap (GE Healthcare). These are a prepacked, single-use gravity-flow column containing precharged Ni Sepharose 6 Fast Flow. The column is intended for purification of histidine-tagged proteins by immobilized metal affinity chromatography (IMAC). Ni Sepharose 6 Fast Flow has high protein binding capacity, low nickel ion (Ni²⁺) leakage and is compatible with denaturing agents plus a wide range of additives. Both clarified and unclarified sample can be applied to the column, and special frits protect the medium from running dry during purification

Solubilised membrane proteins were applied to the prepacked columns at 4°C for 2, previously equilibrated with 20 mM Hepes pH 7.2, 300 mM KCl, 500 μM MgCl₂, 100 μM CaCl₂, 3 mg/ml C₁₂E₈, 0.5 mg/ml Phosphatidylcholine, 15% (v/v) Glycerol, 1 mM DTT.

The columns with the bound protein is then harvested by centrifugation at 1000 rpm in a centrifuge Z383K (Hemle) for 3 min at 4°C, and the flow through was conserved.

The His GraviTrap was washed with the washing buffer (20 mM Hepes pH 7.2, 300 mM KCl, 500 μM MgCl₂, 100 μM CaCl₂, 0.5 mg/ml C₁₂E₈, 0.5 mg/ml Phosphatidylcholine, 15% (v/v) Glycerol, 1 mM DTT and 10 mM Imidazole) and the protein was finally eluted by incubation at 4°C for 30 min rotating in the same washing buffer but with different concentration of Imidazole (25, 50 and 100 mM).

At the eluted protein was added 1.5 mM MgCl₂ and 4.8 μM CaCl₂.

The protein concentration of the samples from the membrane preparation and the protein purification was determined by using the Lowry method.

3.6- PMCA2 preparation for SDS-PAGE analysis

Proteins were separated on SDS-PAGE essentially in accordance with the Laemmli method (Laemmli, 1970). Separating gels of 7.5 or 10 %, depending on protein size, and stacking gels of 5% were used for the electrophoretic separation of proteins. The running buffer contained 25 mM Tris-HCl pH 8.3, 192mM Glycine and 0.1% SDS and the electrophoresis equipment was from Amersham Bioscience. The gels were run at 25 mA for approximately 2-3 hours.

SDS-PAGE was also conducted on NuPAGE®NOVEX pre-cast polyacrylamide mini-gels from Invitrogen. The NuPAGE® System is based upon a Bis-Tris-HCl [Bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl)] buffered (pH 6.4) polyacrylamide gel, with a separating gel that operates at pH 7.0. The NuPAGE MOPS SDS running buffer (20X) contained 50 mM MOPS [[3-(N-morpholino)] propane sulfonic acid], 50 mM Tris base, 0.1% SDS and 1mM EDTA at pH 7. The gels were run at 200 V for approximately 50 min.

SeeBlue Plus2 Pre-Stained Standard (Invitrogen), a mix of 34 proteins with molecular weights from 4 to 250 kDa, was employed for visualizing protein molecular weight range and evaluating transfer efficiency.

Following electrophoresis, the gel was stained with Coomassie Brilliant Blue, allowing visualisation of the separated proteins, or further processed for Western blotting analysis.

3.7- PMCA2 Western Blotting

To detect eventual degradation of PMCA2 during the preparation, all the fractions were analysed by Western Blot, using a PMCA anti-mouse antibody 5F10 (ABR),

Following electrophoresis, the separated proteins were transferred onto PVDF (polyvinylidene difluoride) membranes (Amersham Bioscience) at 350 mA for 2 h in a Blotting buffer containing 47.9 mM TrisHCl pH 8.4, 38.6 mM glycine, 0.0385% SDS and 10% methanol. Blocking of non-specific binding was achieved by placing the membranes for 1h in a saturating buffer (5% non-fat dry milk and 0.1% Tween 20 detergent in PBS). After blocking, a dilute solution of the primary antibody and the membranes were incubated together under gentle agitation for 60-90 min at room temperature, or overnight at 4°C. The membranes were then rinsed for 1h with fresh saturating buffer in order to remove the unbound primary antibody and were incubated for 30-45min with the HRP (horseradish peroxidase)-conjugated secondary antibodies (1:2000 anti-mouse, anti-rabbit or anti-goat IgG HRP, Santa Cruz Biotechnology). The membranes were rinsed with saturating buffer and then with PBS and the chemiluminescence was detected with the ECL techniques (Amersham Biosciences). The signal was captured on Kodak BioMax light films (Sigma).

3.8- Western blotting with anti Calmodulin antibody

The western blotting with anti-Calmodulin (Sigma Aldrich) antibody was performed according Sigma's protocol. The PVDF membranes were incubated for 60 minutes in Fixation Buffer (2% (v/v) glutaraldehyde, freshly prepared in KP Buffer). The membranes were rinsed three times in KP buffer (25 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.0), then incubated in 1 M Lysine for 60 minutes. Blocking of non-specific binding was achieved by placing the membranes overnight in a saturating buffer (5% (w/v) BSA in PBS). After blocking, a dilute solution of the primary antibody (1:1000 in PBS-BSA) and the membranes were incubated together for 2 hours or overnight at 4 °C. The membranes were then rinsed 3 times for 5 minutes each at room temperature using washing buffer (PBS + 0.05% (v/v) TWEEN 20) and were incubated for 1 hour with the HRP (horseradish peroxidase)-conjugated mouse secondary antibody (1:2000).

4- Expression of PMCA pumps in CHO cells

4.1- Continuous cell lines

CHO (Chinese Hamster Ovary) derive from epithelial cell chinese hamster ovary cells and are cultured in 75cm² Falcon flasks in Ham's F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, penicillin (60µg/µl) and streptomycin (120µg/µl)

The cells were maintained in a Haereus incubator at 37°C, in a humid atmosphere containing 5% CO₂ and were split every 2-3 days. When the cells reached 70-80% they were detached from the flask using 0,05% trypsin/0,2%EDTA, in PBS (140mM NaCl, 2mM KCl, 1,5mM KHPO_4 , 8mM Na_2HPO_4 , pH=7,4). Fresh medium was added to the flask in order to inhibit the trypsinization and the cells were collected by centrifugation at 1000 rpm for 3 min, 25°C in a Beckman GS-15R centrifuge (rotor S4180). The pellet was resuspended in 5ml of medium and the cells were seeded onto 13mm glass cover slips or on 24-multiwell plates (for Western blotting analysis) and allowed to grow to 50% confluence. The cells were used for transfection experiments, Western Blot analysis and preparation of membranes cells.

4.2- Transfection

CHO cell lines were transfected using the calcium-phosphate method. One day prior to transfection, the cells were plated onto 150x25 mm dishes such that they were logarithmically growing on the day of transfection (i.e. 30-40% confluent at the time of transfection). 30 µg of plasmid DNA were used for every single dish. The transfection mix was prepared as follows: 25µl of CaCl₂ 2.5 M were added to the plasmid DNA/TE1x 225µl total mix. The tube containing 250µl of HBS 2x (280mM NaCl, 50mM HEPES, 1.5mM Na₂HPO₄, pH 7.12) was gently vortexed and the DNA/TE/CaCl₂ mix was added drop wise. A fine opalescent DNA-calcium-phosphate precipitates formed in about 10 minutes and the transfection mix was then added to the cells (in fresh DMEM growth medium that was changed 2 h before transfection). After 14-16h the cells were washed 2 times with PBS and 1 time with the appropriate growth medium in order to eliminate the DNA-calcium-phosphate precipitates that did not enter the cells. The cells were incubated at 37°C until use.

4.3- Isolation of Microsomes from CHO Cells

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Cells from five 150x25 mm dishes were washed once with 20 ml of phosphate-buffered saline containing 1 mM EDTA and harvested in 10 ml of phosphate-buffered saline containing 0.1 mM phenylmethylsulfonyl fluoride and Cocktail proteases inhibitors EDTA-free (Roche). Cells were collected by centrifugation (2,000 X 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 phenylmethylsulfonyl fluoride, Cocktail proteases inhibitors EDTA-free, and 2 mM dithiothreitol. 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, 2 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, homogenized again with N₂ liquid, and centrifuged at 5,000 X g for 15 min. The supernatant was made 0.6 M in KCl and, in order to remove calmodulin, an excess of EDTA (1.5 mM) was also added. The suspension was centrifuged at 100,000 X g for 40 min to sediment 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 dithiothreitol, and 20 µM CaCl₂, at a protein concentration of 1-3 mg/ml and stored in liquid N₂ until needed.

4.4- Mutagenesis

In vitro site-mutagenesis was carried out with QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions.

5- ATPase activity

The ATPase activity was measured by *Coupled Enzyme Assay*.

The coupled enzyme ATPase assay is based on the conversion of phosphoenolpyruvate (PEP) to pyruvate by pyruvate kinase (PK) coupled to the conversion of pyruvate to lactate by lactate dehydrogenase (LDH). The latter step requires NADH which is oxidized to NAD^+ . NADH absorbs strongly at 340 nm but NAD^+ does not, enabling the utilization of NADH to be followed by monitoring absorbance at 340 nm. The decrease in OD_{340} can be converted into ATPase activity where 1 molecule of NADH oxidized to NAD^+ corresponds to the production of 1 molecule of ADP by the motor ATPase. (**Fig. 2f**)

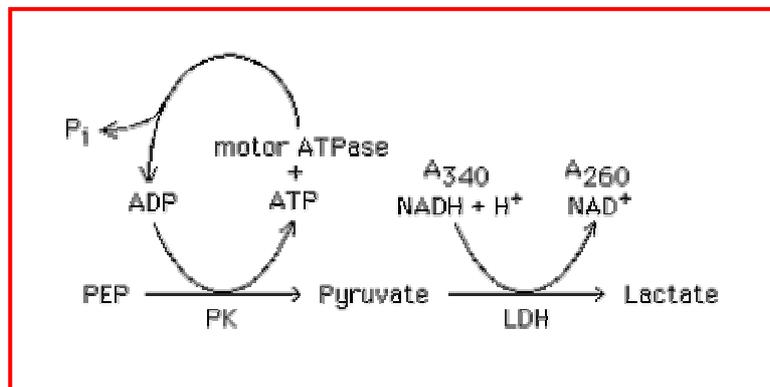


Figure 2f: Coupled Enzyme Assay

The assay was carried out at 37°C in a final volume of 1 ml of a mixture containing precise amounts of reagents reported in the table below:

Reagents	Final Concentration
Tris pH 7.2	20 mM
MgCl ₂	5 mM
EGTA	0.5 mM
KCL	0.1 M
PEP	0.5 mM
NADH	0.15 mM
PK/LDH (Roche)	1.4 U/ml
ATP	40 mM
PMCA Membranes	25 µg/ml
CaCl ₂	50 mM

Table II. Coupled Enzyme Assay Medium components.

The ATPase activity, detected at 340 nm (DU640 Spectrophotometer, Beckman Coulter), is expressed in $\mu\text{mol P}_i/\text{min} \cdot \mu\text{g}$ of Protein (Pyrophosphate moles originated by the ATP hydrolysis): the maximal activity and the basal activity are calculated by multiplication of the activity curve slope value by a factor considering the NADH molar extinction coefficient (ϵ_{NADH}) and the amount of protein (μg). The real activity was obtained by subtracting the basal activity from the maximal activity. The assay was performed in presence of ouabain, thapsigargin and 25 μM PhosphatidylSerine (Sigma Aldrich) and 200 nM Calmodulin (Sigma Aldrich).

6- Immunocytochemistry

Thirty-six hours after transfection, CHO cells were processed for immunofluorescence as follows: they were washed twice with PBS, fixed for 15 min in 3.7% formaldehyde, washed three times with PBS and then incubated for 10 min in PBS supplemented with 50mM NH₄Cl. Permeabilization of membranes was obtained by incubating the cells for 5 min with 0.1% Triton X-100 in PBS, followed by a 1 h wash with 1% BSA (Sigma Aldrich) in PBS. After 2-3 washes with PBS, the cells were incubated for 1h at room temperature in a wet chamber with a primary monoclonal antibody against PMCA (ABCAM) or against GFP (Molecular Probes) , at a 1:100 dilution in PBS. Staining was then carried out with the secondary antibody Alexa Fluor 488 (Molecular Probes) at a 1:200 dilution in PBS. After each incubation, the cells were washed four times with

PBS. The images were acquired using a spinning disk confocal microscope Ultraview (Perkin Elmer Life Sciences) equipped with a 60X oil immersion objective 1.4 N.A.(PlanApo, Nikon).

7- Computational Methods

7.1- Sequence Alignment

A BLAST search performed using the Protein Data Base identify Rabbit SarcoEndoplasmic Reticulum Calcium ATPase (SERCA) pump 1 as the only protein homologous to PMCAs whose structure has been determined. Therefore, the primary sequence of the zb variant of the human PMCA2 (Uniprot entry code: Q01814) was aligned against that of the SERCA1 pump using T-COFFEE (at www.igs.cnrs-mrs.fr/Tcoffee/) (REF Notredame, C., Higgins, D. G., and Heringa, J. (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J.Mol.Biol.* **302**, 205-217) and then manually refined to take into account secondary structure.

7.2- Structural Model.

Based on this sequence alignment, we constructed a comparative modeling-based 3D structure of the PMCA. The model was built with Modeller 8.0 with the automatic loop refinement option (Fiser and Sali 2003a;Fiser and Sali 2003b;Sali and Blundell 1993). Among the different structures available for the SERCA pump we choose the calcium bound form (E1 state, REF Toyoshima, C., Nakasako, M., Nomura, H., Ogawa, H. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. *Nature* v405 pp. 647-655, 2000) PDB entry code:1SU4) since it presents a more open and less structured conformation near the gap corresponding to the insertion of the splicing site A, facilitating the modelling. Residues 35 to 1093 in the sequence of PMCA2 were used to construct a structural model. The selection of the best model was based on the Modeller's objective violation function and WHAT IF analysis (at <http://swift.cmbi.kun.nl/WIWWWI/>) (Hooft, R. W., Vriend, G., Sander, C., and Abola, E. E. (1996). Errors in protein structures. *Nature* **381**, 272). The Ramachandran plot of the selected model gave 91 % of residues in the most favoured zones, and no residues in forbidden region suggesting a geometric and energetically stable structure.

RESULTS

I- Expression and purification of PMCA 2 pump.

Functional properties of an unusual isoform of the plasma membrane calcium ATPase: PMCA 2

Two PMCA pump isoforms (PMCA1 and PMCA4) operate in all tissues and two (PMCA2 and PCMA3) only in specialized tissues, such as muscle and, especially, brain (138). All four isoforms display splice variants caused by the insertion of alternative exons in the primary transcripts: in the translated pump the insertions occur at site A in the cytosolic loop connecting transmembrane domains 2 and 3 and at site C in the C-terminal tail of the pump. Alternative splicing is peculiarly complex in PMCA2 as it involves the insertion of up to three novel exons at site A and of two at site C. The A-site insertions are in-frame, creating variant *w* when three exons are inserted (the normal variant without site- A inserts is termed *z*). The insert at site C creates instead a novel stop codon, leading to the truncation of the pump (variants being the normal full-length pump). The site-C insertions eliminate approximately half of the calmodulin binding domain; those at site A occur next to a domain that binds activatory acidic phospholipids, which, however, also bind to the calmodulin binding domain (67). As expected, the site-C insertions lower the affinity of PMCA pumps for calmodulin (139) but it is still not clear whether it also compromises the activation by acidic phospholipids, which is alternative to that by calmodulin (140). It would be plausible to suggest that the site-A insertion impairs the activation by acidic phospholipids, particularly in the C-terminally truncated pump variants, in which the C-terminal phospholipid binding domain also is compromised.

Several splice variants of the four PMCAs have been detected in cochlear cDNAs (141), but the C-terminally truncated PMCA2*a* being the only isoform detected in the stereocilia of hair cells (98). PMCA1*b* prevails instead in basolateral membranes. Recent work has shown that the truncated isoform in the stereocilia of the outer hair cells (OHCs) is also spliced at site A and is thus the *wa* variant (102). It is plausible to assume that the choice of this unusual variant of the PMCA (92) was dictated by the special Ca²⁺ homeostasis demands of the ambient in which the pump must operate.

We have decided to study the biochemical properties of the PMCA 2 and of its splicing variant (*wa*) present in the outer hair cells of the Corti organ of the inner ear.

The first purpose of this work was the PMCA 2 purification in Baculovirus System and the longer term goal of the project was the preparation of adequate amounts of purified PMCA 2 to attempt crystallization experiments.

The second step was the characterization of the point mutations in the *wa* variant pump that has been shown to induce deafness in mice and humans: thus, they have also been investigated.

1- Expression of the PMCA2 isoform in the Baculovirus system

1.1-Localization of the PMCA 2 *zb* in insect cells

It is known that most secretory and plasma membrane proteins require several processing steps in order to reach the final active form, and that this maturation is essential for the correct transport of the protein to its final destination. To investigate if the PMCA2 *zb* protein was positioned in the plasma membrane, Sf9 cells were infected with the PMCA2 *zb* Baculovirus. After 48h cells were fixed in paraformaldehyde and incubated with the following antibodies: monoclonal antibody against all the PMCA isoforms (5F10) and polyclonal antibody against the PMCA2 pump. A FITC conjugated secondary antibody and Alexia 594 were used to produce the fluorescence (**Fig. 3c**). In infected cells both 5F10 and 2N recognized the Calcium pump. The infection score was more or less 80%.

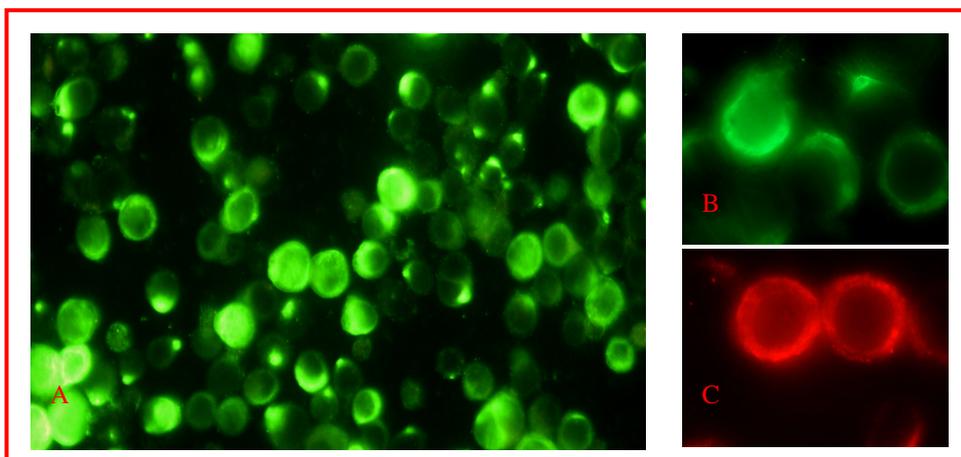


Figure 3c: Immunofluorescence localization of 5F10 and 2N reactive proteins in Sf9 insect cells **A.** 5F10 labeled cells infected with PMCA2 *zb* Baculovirus, showing that the calcium pump is localized preferentially in the plasma membrane **B.** Image of the 5F10 antibody with 40X objective in which was possible to appreciate the membrane localization. **C.** Immunofluorescence membrane localization with 2N antibody.

1.2- Expression and purification of PMCA 2 *zb*

After that PMCA 2 membrane localization and successfully infection was confirmed; crude membranes from Sf9 cells infected with PMCA 2 *zb* baculovirus for 72 hours were prepared and solubilized; their proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. In **Fig. 3d** band of molecular mass of approximately 135-140 kDa was observed in membranes preparation with/without calcium. The membrane fractions were richer in PMCA2 protein than total cells.

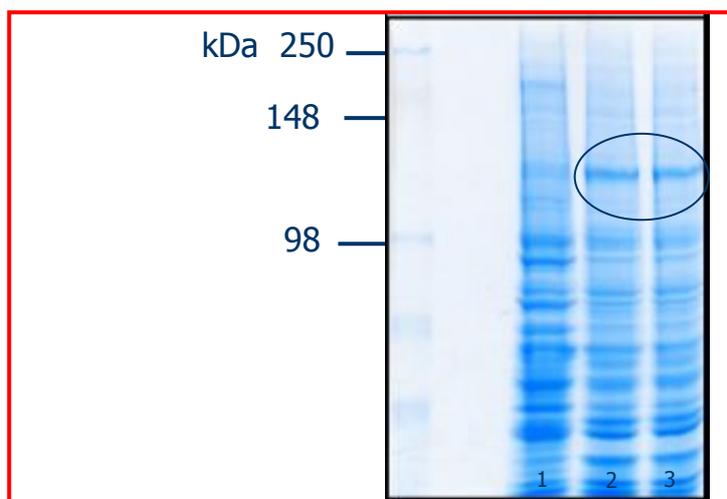


Figure 3 d: Expression of the PMCA 2 isoforms. The membranes were isolated and separated in 7.5 % SDS-PAGE gel 1. Total sf9 cells 2. Membranes prepared without Ca^{2+} 3. Sf9 Membranes prepared with calcium. The blue circle shows the PMCA 2 *zb* expression.

1.3- Time course of PMCA 2 *zb* expression in Sf9 cells

A PMCA 2 *zb* expression time course was performed to analyze the time-dependent dimerization and degradation of PMCA in the Sf9 insect cells. Avoiding dimerization and degradation is crucial for PMCA binding to the Calmodulin resin for affinity chromatography purification. Sf9 cells infected with Baculovirus were then collected at several infection times and the protein concentration was assayed with the Lowry et al. method (142) (**Table III**).

Infection times	Protein concentration
12 hours	6.4 $\mu\text{g}/\mu\text{l}$
18 hours	6.8 $\mu\text{g}/\mu\text{l}$
24 hours	7.4 $\mu\text{g}/\mu\text{l}$
36 hours	7.8 $\mu\text{g}/\mu\text{l}$
40 hours	8.2 $\mu\text{g}/\mu\text{l}$

TABLE III: PMCA2 *zb* expression time course protein determination

The PMCA 2 *zb* expression was then checked by Western blotting with an antibody against the PMCA (Fig 3e). PMCA 2 pump appears to be strongly degraded at 40 hours post infection while at 12 and 18 hours non degradation and dimerization were observed. Evidently, as the expression time increased Sf9 cells develop proteolytic systems that degrade the recombinant PMCA 2. An expression time of 18 hours was thus chosen for the infection prior to the calmodulin affinity purification.

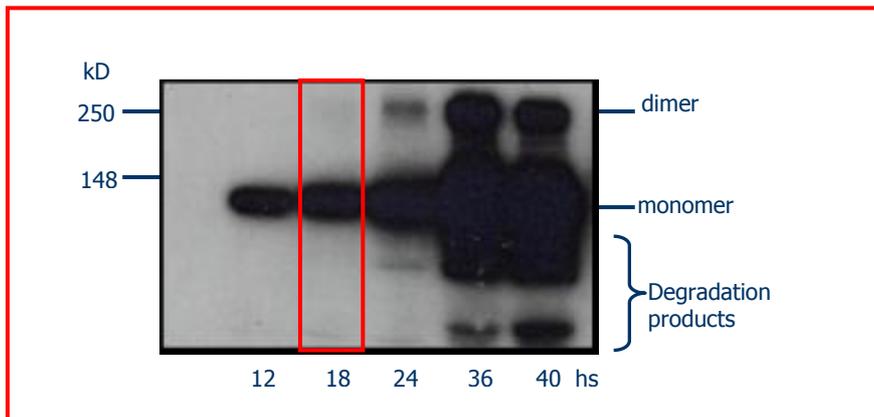


Figure 3e: Time course of the Sf9 insect cells infection to determine the level of the expression of PMCA 2 *z/b*. In this figure were indicated the times of infection, the monomer and dimmers of PMCA 2, degradation products were observed after 24 hours post infection.

1.4- Preparation of Sf 9 cells membranes for purification with a calmodulin resin

The membranes prepared from Sf9 cells infected with recombinant baculovirus were assayed with the Lowry method for protein determination. The protein concentrations of different membranes fractions are represented in the **TABLE IV**

Preparation membrane Fraction	Protein concentration
Total Cells	20 µg/µl
Supernatant homogenized	10,3 µg/µl
Membranes	9,1 µg/µl

TABLE IV: Sf9 membranes protein determination

The fractions were then checked by Western blotting with an anti PMCA 2 antibody (**Fig. 3f**). It was confirmed that after 18 hours of infection, the degradation of PMCA 2 *zb* in the membrane fraction is prevented.

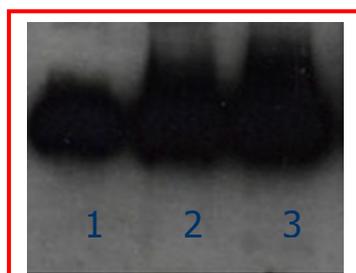


Figure 3f: Immunoblotting of the Sf9 membranes preparation fractions with anti PMCA antibody 1.Total cells; 2. Supernatant homogenized; 3.Total membranes

1.5- PMCA 2 *zb* solubilization and purification by a calmodulin affinity resin.

Membranes prepared from Sf9 cells infected with recombinant baculovirus contained a major band at 135 kDa corresponding to PMCA 2 protein. The membranes containing the pump isoform were solubilized with a non-ionic detergent C₁₂ E₈ at a ratio 1:1. A calmodulin Sepharose resin was then used. The procedure removed most unrelated proteins, but contaminants were still visible in Colloidal Coomassie gels of the EDTA eluates (**Fig 3g A**).The protein concentrations was determined by the method of Lowry (**TABLE V**)

Solubilized Fraction	Protein concentration
Membranes solubilized	5 µg/µl
Flow Through	3,3 µg/µl
Elution I 2 mM EDTA	2,75 µg/µl
Elution II 2 mM EDTA	2,8 µg/µl

TABLE V: Membranes solubilization and Calmodulin affinity fractions protein determination

All fractions were subjected to SDS-PAGE and stained with Colloidal Coomassie, but proteins in the eluted fractions were only visible after concentration with Vivaspin concentrator tube. (**Fig 3g B**)

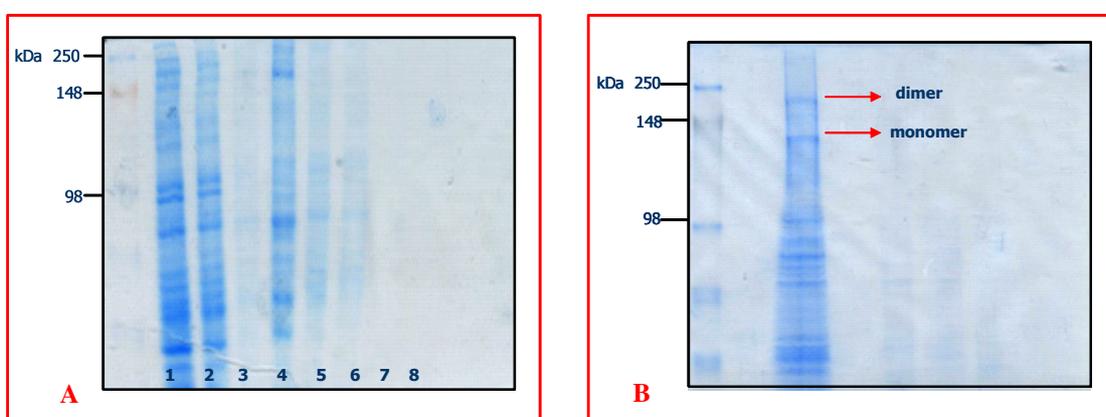


Figure 3g: Partial purification of the recombinant PMCA 2 by Calmodulin Affinity

A. 1. Total cells; 2. Supernatant Homog. ; 3. Membranes 4. Pellet solub.; 5- Super. Solub.; 6. FT; 7. elution I 2mM EDTA; 8. elution II 2mM EDTA **B.** Elution I and II after vivaspin concentration

To verify the presence of PMCA 2 in the eluates, Western blotting analysis was performed using an anti PMCA antibody (**Fig. 3h**)

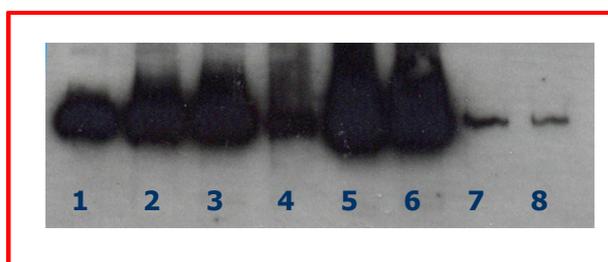


Figure 3h: Western blotting analysis of the partial purification of the rPMCA 2 by Calmodulin affinity column. 1.Total cells; 2.Supernatant Homog.; 3.Membranes; 4. Pellet solub.; 5.Super. Solub.; 6.FT; 7.elution I 2mM EDTA; 8.elution II 2mM EDTA

The level of PMCA expression was low, as expected, occurring to the lower time of infection, but the PMCA binding to the calmodulin resin was also poorly efficient. PMCA 2 *zb* seemed not to be come bound to the calmodulin, appearing almost completely in the FT fraction. This was not expected, given the high affinity, of PMCA 2, for calmodulin. The reasons for this abnormal behavior are now investigated, thus, we decided to investigate the binding of calmodulin to the partially purified PMCA 2 pump, estimated on PVDF membranes by exposing them to a calmodulin antibody. A typical experiment is shown in **Fig. 3i**.

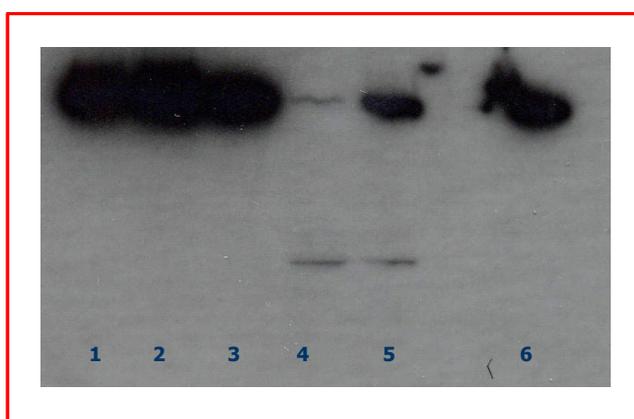


Figure 3i: PMCA2 is binding to Calmodulin in Sf 9 insect cells. Western blot analysis was performed with anti calmodulin antibody in different membrane fractions. 1. Membranes solubilized $-Ca^{2+}$; 2. Membranes solubilized $+Ca^{2+}$; 3. Membranes; 4. Membranes with 10mM EDTA; 5. Supernatant homogenized; 6. Pellet homogenized

These data, let us conclude that PMCA 2 has an extremely high calmodulin affinity, thus, it may probably be calmodulin, saturated within the Sf9 cells and in the partially purified state. The high affinity of PMCA 2 for calmodulin evidently complicates its purification using Calmodulin affinity.

1.6- Construction of a Recombinant Baculovirus with a His-Tag Vector

The complete full length PMCA 2 *zb* and *wa* spliced isoforms were subcloned into a Baculovirus shuttle fusion vector pFastBacHtB (fusion with the N-terminal 6xHis tag and the TEV protease cleavage site). The pump was digested with Hind III from a pcDNA3 plasmid in the case of *zb* splice form, and XhoI/NotI in the case of *wa* splice variant.

The baculoviruses, in both cases, were produced as described above.

1.7- Time course PMCA 2 *wa* expression in Sf9 cells

A PMCA 2 *wa* expression time course was performed to analyze the time-dependent dimerization and degradation of PMCA in Sf9 cells. Sf9 cells infected with Baculovirus were then collected at several infection times and the protein concentration was assayed (Table VI).

Infection times	Protein concentrations
18 hours	3.8 $\mu\text{g}/\mu\text{l}$
24 hours	4.4 $\mu\text{g}/\mu\text{l}$
36 hours	7.8 $\mu\text{g}/\mu\text{l}$
48 hours	8.2 $\mu\text{g}/\mu\text{l}$

TABLE VI: PMCA2 *wa* expression time course protein determination

The PMCA 2 *wa* expression was then checked by Western blotting with an antibody anti PMCA and with an anti Histidine Tag antibody (Fig 3j). PMCA 2 pump dimers appears after 24 hours post infection while at 18 hours the protein was detected only by anti His-Tag antibody. Dimerization was observed at any post infection time. A band of 90 kDa was detected by the anti His-Tag antibody but not with that against the PMCA, possibly because this antibody recognized a protein rich in Histidine. An expression time of 24 hours was chosen prior to the insect cells infection for the His-Tag affinity purification

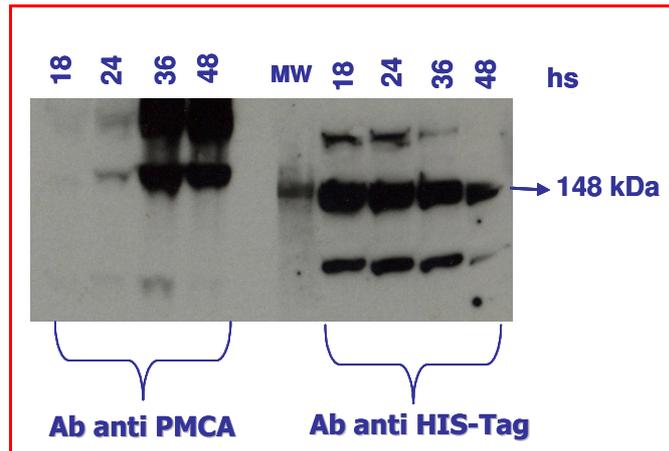


Figure 3j: Time course of the Sf9 insect cells infection to determine the level of the expression of PMCA 2 *wa*. The membranes proteins were separated in SDS-PAGE gel and probed with 5F10 (anti PMCA) and ant His-Tag antibody

1.8- Sf 9 insect cells membrane preparation for His-Tag affinity purification

The Sf9 cell membranes were prepared and solubilized in the C₁₂ E₈ detergent as described above. The fractions infected with recombinant baculovirus PMCA 2 *zb* and *wa* were then assayed for the protein content. The protein concentrations of different membrane fractions are represented in the **TABLE VII**

Preparation membrane Fraction	Protein concentration	
	PMCA 2 <i>zb</i>	PMCA 2 <i>wa</i>
Membranes	9,1 µg/µl	12,5µg/µl
Membranes solub.	5,8 µg/µl	5,3 µg/µl
Pellet after solub.	7,8 µg/µl	9,1 µg/µl

TABLE VII: Sf9 membranes protein determination

The fractions were then checked by Western blotting technique with the anti PMCA 2 antibody (**Fig. 3k**). No degradation was detected after 24 hours of infection with either the PMCA 2 *zb* and *wa*, however, some dimerization was present.

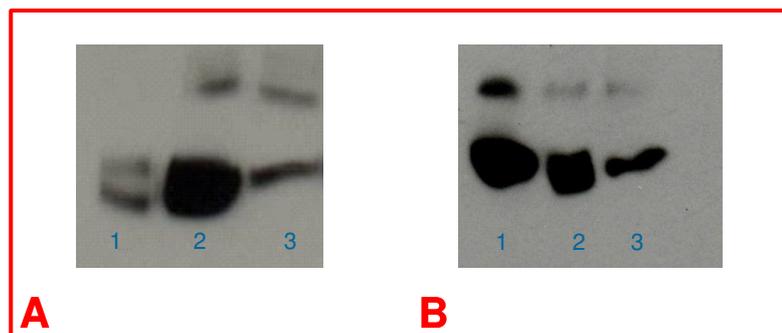


Figure 3k: Immunoblotting of the Sf9 membranes preparation fractions with anti PMCA antibody A.PMCA 2 *zb* B.PMCA 2 *wa* 1.Membranes; 2.Membranes solubilized; 3.Pellet after solubilization

1.9- PMCA 2 *zb* and *wa* purification by His-Tag affinity column.

The pump splicing isoforms were solubilized with the non-ionic detergent C₁₂ E₈ at a ratio 1:1. Purification was attended on solubilized membranes using a His-Tag spin column. The procedure removed most unrelated proteins, but numerous contaminants were still visible in Colloidal Coomassie gels of the imidazole eluates at different concentrations (Fig 3l).The protein concentrations were determined by the method of Lowry et al (TABLE VIII)

Preparation membrane Fraction	Protein concentration	
	PMCA 2 <i>zb</i>	PMCA 2 <i>wa</i>
FT	4,1 µg/µl	4,5µg/µl
Elution 25mM Imidazole	2,8 µg/µl	2,3 µg/µl
Elution 50mM Imidazole	2,5 µg/µl	2,2 µg/µl
Elution 100mM Imidazole	1,8 µg/µl	2,1 µg/µl

TABLE VIII: His-Tag affinity fractions for protein determination

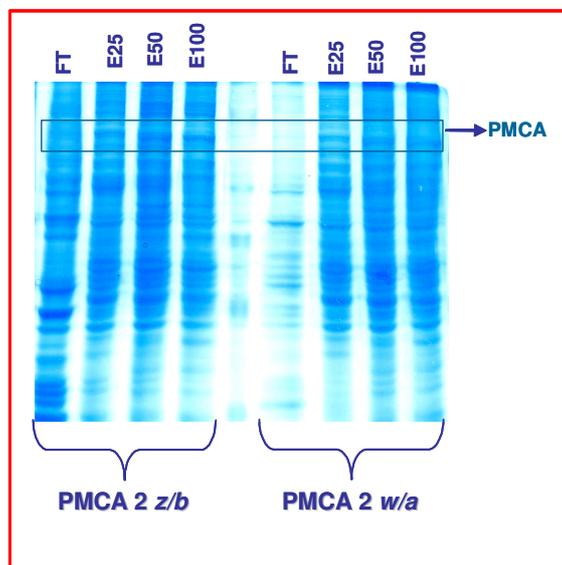


Figure 3l: Partial purification of recombinant PMCA 2 by His-Tag Affinity

To verify the presence of PMCA 2 in the gel, Western blotting analysis was performed using the anti PMCA antibody (Fig. 3m)

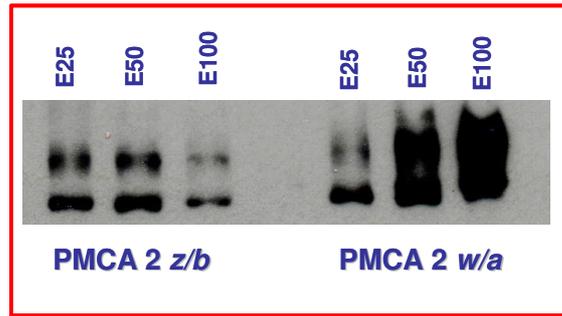


Figure 3m: Western blotting analysis of the partial purification of the PMCA 2 by Histidine Tag affinity column.

In order to characterize the PMCA 2 pump and facilitate the purification, we expressed an N-terminal 6XHis-tagged version of the ATPase pump. The PMCA2 was immunodetected as a band of about 135 kDa localized in the plasma membrane of Sf9 insect cells. The purification of the PMCA 2 *z/b* and *wa* splicing pumps was unsuccessful. The principal problem was a low level of expression and the low time of infection in the insect cells. PMCA 2 degradation and dimerization were found after 24 hours of infection, perhaps long time of infection increases the level of expression but the pump is degraded by calpains and other proteases that attack the C-Terminal of the pump.

II- Biochemical study of mutants of plasma membrane calcium pump isoform 2 causing deafness

Mutations of PMCA2 have been recently shown to induce recessively inherited deafness (**86**): in the first described mouse a G283S mutation replaced a conserved G downstream of the phospholipids binding domains of the pump (deafwaddler mouse, *dfw*). A mutation next to the active center (V586M) was later found to depress pump activity and to increase hearing loss in heterozygous patients that also carried a homozygous mutation in cadherin 23 (CDH23) (**121**). A deafness inducing mutation has then been identified in the human PMCA2 gene that replaces a conserved glycine residue (G293S) 10 residues downstream of the *dfw* mutation.

Within the stereocilia, the control of Ca^{2+} is vital to a number of aspects of the mechanotransduction process, e.g., the regulation of adaptation, the ability to sense the deflection of the ciliary bundle with high sensitivity, and the breaking and regeneration of tip links. The peculiar functions of the Ca^{2+} signal have in all likelihood dictated the choice of the PMCA 2 *wa* isoform. Because endolymph Ca^{2+} is very low, it would make sense to control it with a stereociliary pump variant that ejects Ca^{2+} less efficiently than other isoforms.

In the second part of this work, the activity of the recombinant *wa* variant of the PMCA2 pump was thus compared in a Baculovirus expression system with the *zb* variant of the pump and with the *dfw* pump and the pump carrying the G293S human mutation. The splicing variants of the pump and the mutations have been studied biochemically by a coupled enzyme assay.

A novel mutation (Oblivion) was also studied; this mutation is localized in the sixth transmembrane domain of the PMCA 2 *wa* pump where a Serine is replaced by a Phenylalanine at position 877. The Oblivion mice present ataxia, loss of balance and progressive loss of the auditory function.

1- Construction and expression of the PMCA2 *wa* mutants

The experiments were particularly aimed at understanding the biochemical properties of the mutants, of the *wa* splice variant of the PMCA 2 pump which is the resident isoform of the stereocilia. The mutagenesis was performed by site-directed mutagenesis and the cassettes containing the mutations were sequenced to completion in at least one direction. The mutated PMCA 2 pumps were expressed in Sf9 insect cells using the Baculovirus System. The mutants, the wild type stereociliary pump (*wa*) and the PMCA 2 *zb* pump used as a control were expressed at about the same levels, indicating that the mutations had not increased the propensity of the pump to become proteolyzed.

In crude membranes of Sf9 cells, a band migrating with a molecular mass of 135 kDa was recognized by the PMCA2 antibody. (Fig. 3n)

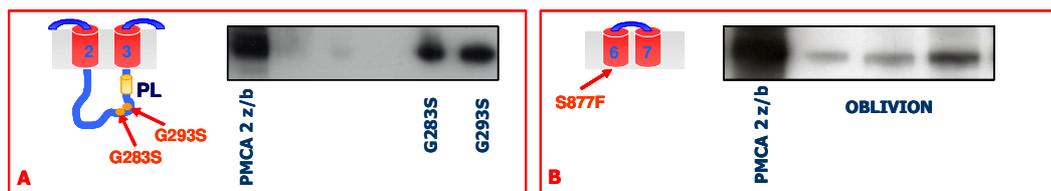


Figure 3n: Transient expression of the mutated PMCA 2 *wa* pumps in Sf9 insect cells. A and B, 10 μ g of crude membrane proteins prepared by freeze and thaw method, were separated by SDS-PAGE, transferred to PVDF membranes and blotting with antibody specific for PMCA 2 pump. In the panel A the antibody recognizes the dfw mutant (G283S) and the human mutation (G293S). In panel B Oblivion mutation was also stained by PMCA 2 antibody. PMCA 2 *zb* was used as a control.

1.1-Determination of the Ca^{2+} transport activities of microsomes from PMCA 2 *zb*, PMCA 2 *wa* and of mutated pumps of the *wa* variant.

Microsomal membranes isolated from PMCA 2 *zb*, PMCA 2 *wa*, PMCA 2 G283S, G293S and Oblivion infected cells were assayed in the presence of thapsigargin and oligomycin to inhibit the activity of the endogenous endoplasmic reticulum Ca^{2+} pump and the mitochondrial ATP mediated Ca^{2+} uptake system (143). In Sf9 cells membranes expressing the PMCA 2 *zb*, the Ca^{2+} ATPase was higher than in those of cells expressing the PMCA 2 *wa* pump. The specific activity of the G283S mice pump

mutant and G293S human mutant were almost the same but were lower than that of the wild type *wa* pump. The concentration level of the protein expression was verified by Western blotting. (Fig. 3o)

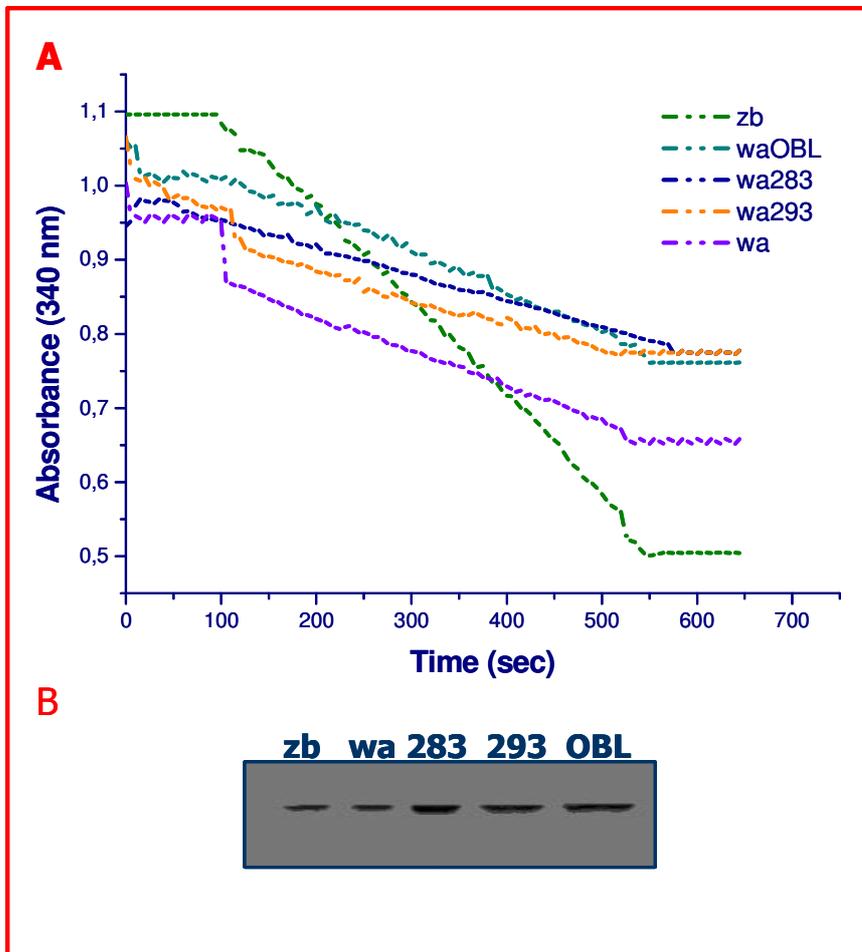


Figure 3o: Comparison of Ca²⁺ uptake activities of microsomes isolated from PMCA 2 zb, PMCA 2 wa, PMCA 2 wa G283S, PMCA 2 wa G293S and PMCA 2 wa Oblivion infected Sf9 cells. A. The Ca²⁺ ATPase activity was measured spectrophotometrically using coupled enzyme assay. The medium contained 120 mM KCl, 60 mM Hepes, 1mM MgCl₂, 2.5 mM ATP, 0.2 mM NADH, 0.5 mM PK/PL, 1 unit of LDH and 20 mM EGTA. The assay was performed in presence of oligomycin and thapsigargin. **B.** Western blotting assay using PMCA 2 antibody

The calcium uptake activity (measured as ATP splitting) was calculated as Δ Absorbance / Δ time from each curve and it is represented in the **Figure 3p**. The error bar represents the standard errors of the mean.

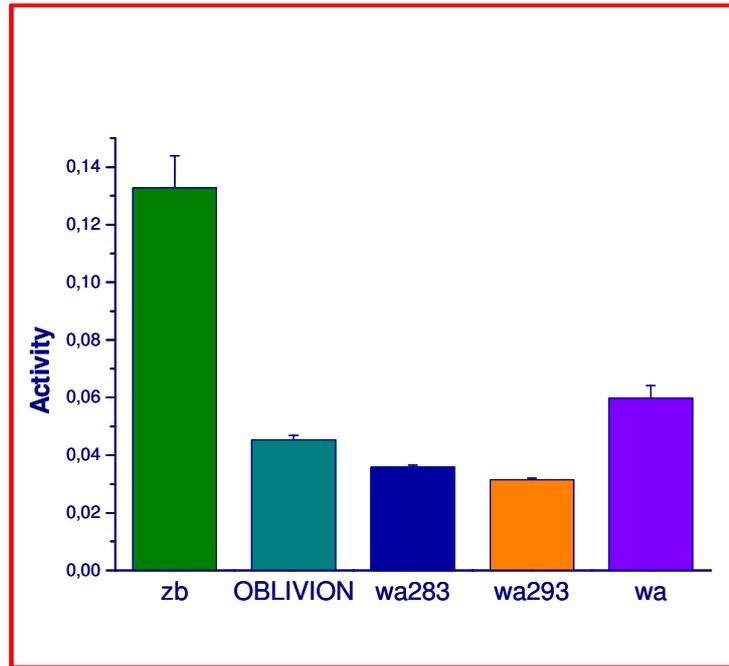


Figure 3p: The Ca^{2+} uptake activity of the PMCA2 *zb*, *wa* and mutants pumps. The Ca^{2+} activity of the PMCA 2 *zb* pump was almost 2 fold higher than PMCA2 *wa*. The *wa* 283 and *wa* 293 had lower activity than wild type pump. At the 0,05 level the means are significantly different.

These data suggest that, the calcium transport activity defect with respect to the wild type *wa* variant was more pronounced in the *dfw* mutant (G283S) than in the G293S (human) mutant. The PMCA 2 of the Oblivion mouse was slightly more active than the pumps bearing the 283 and 293 mutations but still much lower that of the wild type.

1.3- Study of a novel mutation near to the ATP binding domain of the PMCA 2 pump.

Another mutation (2075G→A) results in the substitution of a methionine for a valine at amino acid position 586 (V586M) in the T4–T5 intracellular catalytic loop of PMCA2. Molecular modeling of V586M based on the template of the three-dimensional structure of the closely related sarcoplasmic reticulum calcium pump predicts that substitution with the sterically larger methionine side chain would distort packing underneath the ATP-binding interface or increase its projection from the external solvent-exposed surface of the nucleotide-binding domain. The valine residue at position 586 is completely conserved among mouse, rat, and fish PMCA2 orthologues, and either valine or a conservatively substituted residue (isoleucine) are present at this position in

all known PMCA1 and PMCA3 amino acid sequences. The V586M mutation was detected in a mouse, called Tommy, that were affected by sensorineural hearing loss, suggesting that this variant may modify the severity of sensorineural hearing loss caused by a variety of factors.

Crude membranes from CHO cells transfected with a cDNA encoding the 2 *zb*, PMCA2 *wa* and Tommy (V586M) variant were prepared and separated by SDS-polyacrylamide gel. The proteins were analyzed by Western blotting and stained by Coomassie Brilliant Blue. (**Fig. 3q**)

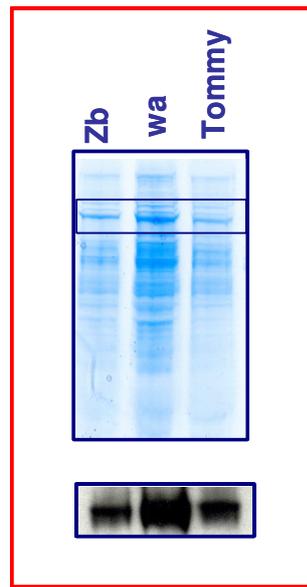


Figure 3q: Coomassie and Western Blotting analysis of PMCA 2 *zb*, *wa* and Tommy pumps

Microsomal membranes isolated from CHO cells transfected with PMCA 2 *zb*, PMCA2 *wa* and Tommy (V586M) mutants were assayed in the presence of thapsigargin and oligomycin. PMCA 2 *zb* had higher reactivity to Ca^{2+} than PMCA 2 *wa*. When expressed in CHO cells, PMCA2 Tommy variant had approximately 25 percent of the calcium stimulated ATPase activity of the wild-type PMCA 2 *wa*. (**Fig.3r**)

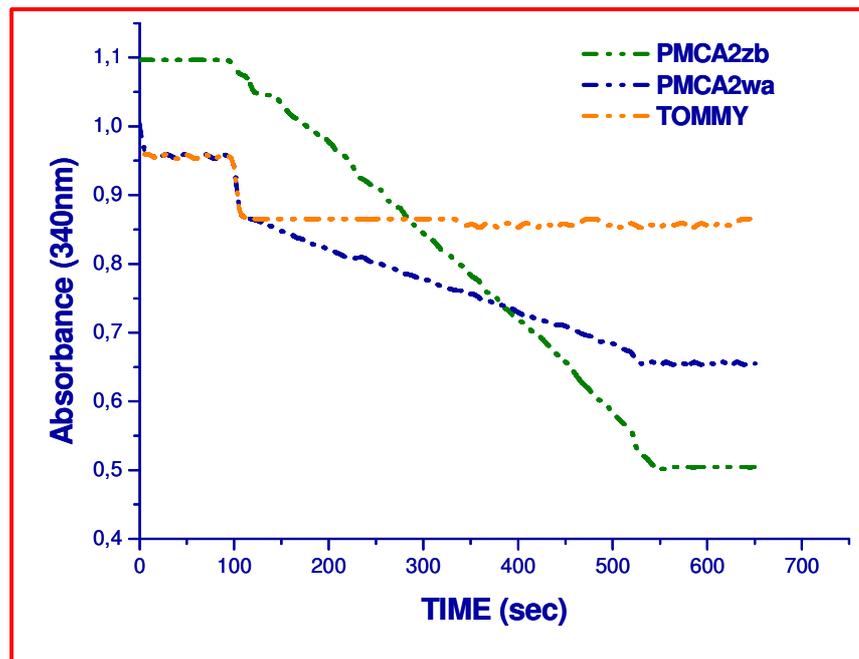


Figure 3r: The Ca^{2+} uptake activity of the PMCA2 *zb*, *wa* and Tommy mutant. The calcium ATPase activity of PMCA2 *zb*, PMCA2 *wa* and Tommy was determined in the absence of calmodulin. Three ATPase experiments were performed for each expressed protein from three microsomal preparations of three different expressed-protein preparations. Representative data are shown from one of the three experiments.

III- Functional properties of the PMCA 2 *wa* splice variant.

The PMCA2 isoforms (the products of gene ATP2B2) are transcribed in a limited number of tissues, chiefly, brain and skeletal muscle (78, 95,100). Their sequences have been deduced independently by different groups (78, 79, 82), and their human gene has been mapped to chromosome 3 (3p25-26) (78, 79, 80), some 200-500 kilobase pairs away from the locus containing the gene associated with the von Hippel-Lindau disease (79). Specific brain regions, e.g. the Purkinje's cells layer, contain high levels of PMCA 2 transcripts (87).

During the isolation of human clones for the PMCA2 pump an alternative splicing process was discovered in the N-terminal half of the protein (82). The C-terminal splicing of PMCA transcripts had been described previously (93). The 1 to 3 exons will insert in the transcript (95) (splicing site A). The insertion occurred in the region corresponding to the mid portion of the cytosolic loop between transmembrane domains 2 and 3. A region of 65 amino acids just downstream of this splicing site was suggested to modulate the activation of the PMCA pump by the calmodulin binding domain (46), and a region upstream of it was proposed to interact with activating acidic phospholipids (51).

The aim of this chapter was the study of the properties of the PMCA 2 and its splicing products, represented in the **Figure 3s**

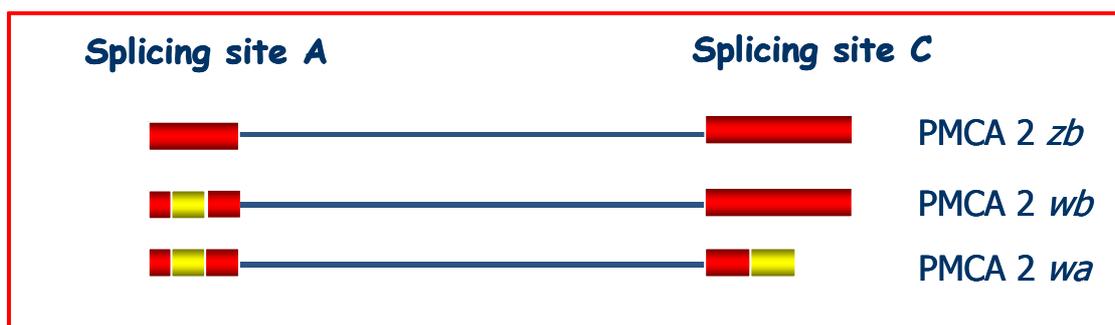


Figure 3s: Schematic representation of the PMCA 2 splicing variants

1- Functional studies of PMCA *wa*. Unusual variant of PMCA 2 pump.

1.1- Expression of PMCA 2 splicing variants in CHO cells

To study the Ca^{2+} transporting activity of the PMCA 2 splice variants *zb*, *wb*, *wa* and that of the mutant Tommy PMCA *wa* pumps, the full-length clones were overexpressed in CHO cells. Crude membranes from cells transfected with cDNA encoding the splicing variants and the mutant were prepared and their protein (1 μg) separated by a 7.5% SDS-polyacrylamide gel. **Fig. 3t** shows a Western blot using the 5F10 antibody and gene reporter GFP monoclonal antibodies to visualize the variants and the mutant. The estimated molecular weights corresponded to the expected molecular masses of each isoform based on their protein sequences. The level of expression was about the same for all four isoforms.

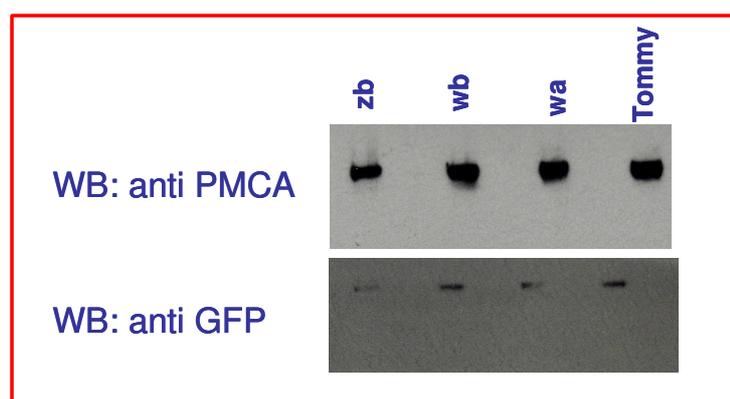


Figure 3t: Immunoblot of microsomes prepared from CHO cells transfected with plasmid encoding GFP and pumps proteins

1.2- Determination of Ca^{2+} Transport Activities of Microsomes from transfected CHO Cells with PMCA 2 *zb*, PMCA 2 *wb*, PMCA 2 *wa* and PMCA 2 *wa* Tommy.

Microsomal membranes isolated from cells transfected with PMCA 2 *zb*, PMCA 2 *wb*, PMCA 2 *wa* and PMCA 2 *wa* (Tommy) were assayed in the presence of thapsigargin and oligomycin to inhibit the activity of the endogenous endoplasmic reticulum Ca^{2+} pump and the ATP linked mitochondrial Ca^{2+} uptake as described above. **Figure 3u** shows Ca^{2+} uptake by the microsomal in the absence of calmodulin. Both PMCA 2 *zb*

and PMCA 2 *wb* had higher basal activity than PMCA 2 *wa*. PMCA 2 *wa* (Tommy) only had about 25 per cent of the activity of PMCA 2 *wa* (see previous section).

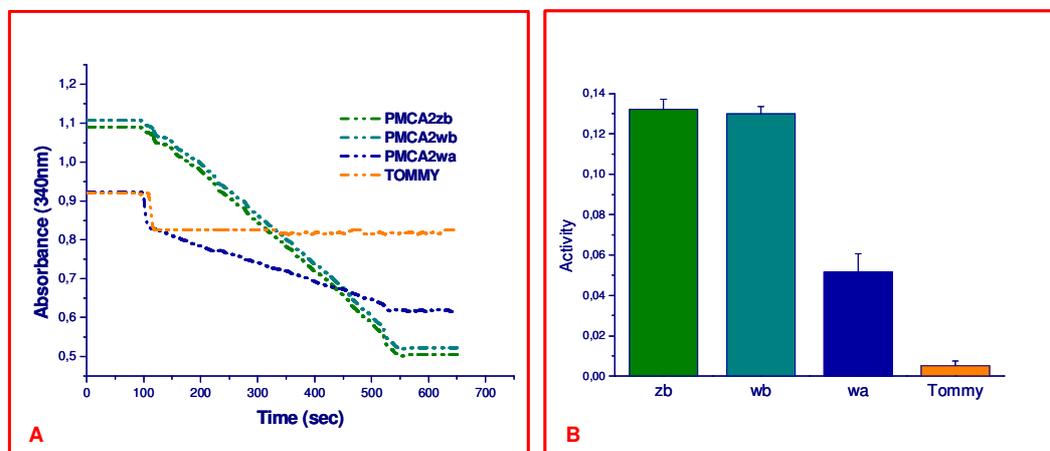


Figure 3u: Comparison of Ca^{2+} activities of microsomes isolated from PMCA 2 *zb*, PMCA 2 *wb*, PMCA 2 *wa* and PMCA 2 *wa* Tommy pumps transfected CHO cells. A. Activity of recombinant PMCA2 isoforms and of the *wa* isoforms in CHO cells. CHO cells were transiently transfected with the PMCA2 variants. Membrane vesicles were preincubated at 37 °C and Ca^{2+} uptake was initiated by the addition of ATP. The histograms show the means \pm SD of the activity of the pumps. The traces are representative of at least three experiments with different membranes preparations. At the 0,05 level the means are significantly different.

1.3- Determination of Ca^{2+} Transport Activities of Microsomes from CHO Cells transfected with PMCA 2 *zb*, PMCA 2 *wb*, PMCA 2 *wa* and PMCA 2 *wa* Tommy transfected. Calmodulin activation

The calmodulin sensitivity of each isoform was observed by measuring its Ca^{2+} transport activities at a fixed Ca^{2+} concentration in the presence of the 200 nM calmodulin (Fig. 3v). PMCA2 *zb* and *wb* showed the highest response to calmodulin i.e., the activity of these isoforms was over eight times higher than of PMCA 2 *wa*. PMCA2 *wa* had lower affinity for calmodulin than PMCA2 *zb* and *wb*, but had still higher affinity than the corresponding Tommy mutant.

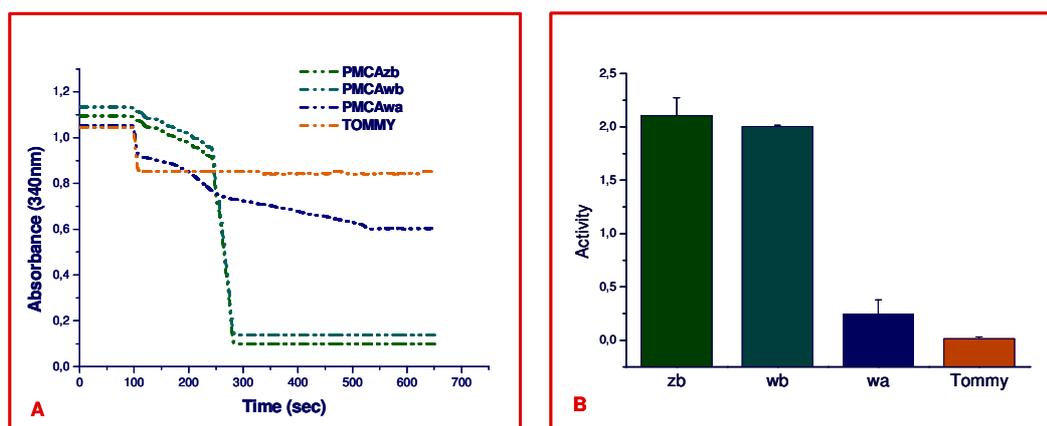


Figure 4v: Fig. 2. PMCA 2 *zb* and *wb* are more sensitive to calmodulin stimulation than PMCA *wa*. **A.** The calmodulin dependence of Ca^{2+} uptake by microsomal vesicles isolated from CHO cells transfected with PMCA 2 *zb* (green curve), PMCA 2 *wb* (light blue), PMCA 2 *wa* (blue) and Tommy (orange) is shown. Membrane vesicles were preincubated at 37 °C with 200 nM of calmodulin and Ca^{2+} uptake was initiated by the addition of ATP. **B.** The histograms show the means \pm SD of the activity of the pumps. At the 0,05 level the means are significantly different

1.4- Determination of Ca^{2+} Transport Activities of Microsomes from CHO Cells transfected with PMCA 2 *zb*, PMCA 2 *wb*, PMCA 2 *wa* and PMCA 2 *wa* Tommy. Activation by Phosphatidylserine

Acidic phospholipids bind to the PMCA protein and activate it by interacting with two domains, the calmodulin binding domain and a sequence close to splicing site A (51).

The splicing event that occurs at site A affects the sequence immediately 5' to the region that encodes one of the phospholipid-sensitive portions of the PMCA. Acidic phospholipids, in particular polyphosphoinositides, are potent activators of the PMCA. As mentioned, splice site A is situated between the phospholipid binding region and a sequence further downstream that is involved in an intramolecular inhibitory interaction with the C-terminal calmodulin binding domain

Data showing that the Ca^{2+} -calmodulin-stimulated pump (or the truncated *a* variant of the pump lacking the COOH-terminal autoinhibitory, calmodulin binding domain) could be further stimulated by phospholipids indicated that a region(s) other than the COOH-terminal domain which also binds acidic phospholipids must also be involved in the lipid stimulation. The mechanism by which different phospholipids activate the PMCA is not known. However, given the location of the lipid-binding sequences in the pump,

one speculated that the interaction of acidic phospholipids (presumably via their charged headgroups) with the calmodulin binding domain leads to some structural rearrangement that weakens the autoinhibitory intramolecular interactions formed by the COOH-terminal tail

The Ca^{2+} -dependent ATPase associated with CHO membranes of cells expressing the PMCA 2 *zb*, *wb*, *wa* and *wa* Tommy was stimulated by phosphatidyl serine. The PMCA 2 *zb* and *wb* had the same response to this acidic phospholipids implying that the inserts next to the N-terminal phospholipids binding domain (variant *w*) was not disturbed by the splicing insert. The PMCA *wa* was absolutely not stimulated by phosphatidyl serine (**Fig. 4w**): apparently, then, no activation by acidic phospholipids can occur when the C-terminal binding site is undisturbed. The response of the PMCA 2 *b* pumps to PS was 4 fold higher than that of the *wa* variant.

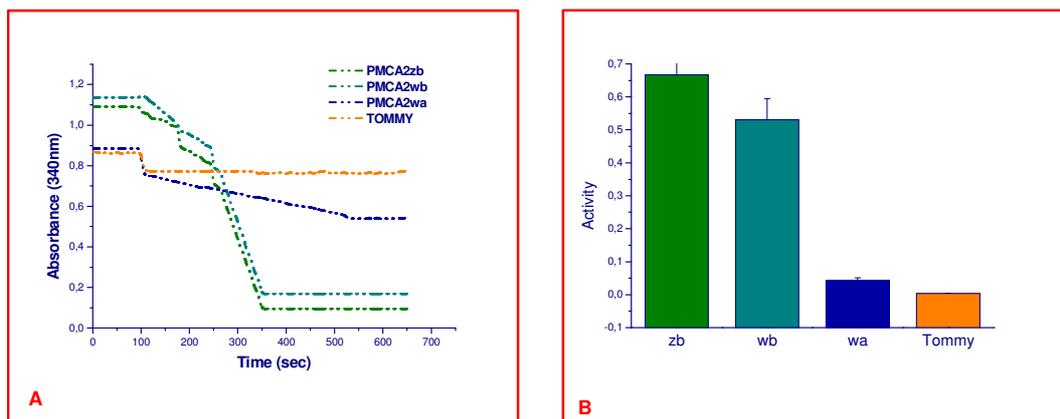


Figure 4w: Stimulation of the Ca^{2+} -ATPase by acidic phospholipid **A.** The PS dependence of Ca^{2+} uptake by microsomal vesicles isolated from CHO cells transfected with PMCA 2 *zb* (green curve), PMCA 2 *wb* (light blue), PMCA 2 *wa* (blue) and Tommy (orange) is shown. Membrane vesicles were preincubated at 37 °C with 25 μM PS and Ca^{2+} uptake was initiated by the addition of ATP. **B.** The histograms show the means \pm SD of the activity of the pumps. At the 0,05 level the means are significantly different

IV- Structural model of PMCA 2 and a biochemical study of the effect of a Met265Ala mutation in one of the domain that mediate the auto-inhibition of the pump

1- Comparative model between SERCA and PMCA 2

To investigate whether CaM indeed removes its binding domain from the cytosolic loops of the pump, we decide to introduce a mutation in one of the two sites that interact with the CaM binding domain in the cytosolic loops of the pump. Prior to this, the PMCA pump has been modeled to decide which residue to mutate. The sequence alignment presented in **Fig. 3x** illustrates the degree residues conservation among the PMCA and the SERCA pump template sequences. Both sequences align with relatively low conservation showing gaps and insertions, among which the largest correspond to the splicing site A. Also as expected, the phosphorylation site is well conserved while the residues involved in the recognition of Thapsigargin (**144**) by the SERCA pump are not conserved in the PMCA pumps. In agreement with the fact that this compound does not inhibit PMCA pumps.

Based on this sequence alignment, we have constructed a comparative modeling-based 3D structure of the PMCA. The model was built with Modeller 8.0 with the automatic loop refinement option. Among the different structures available for the SERCA pump we chose the calcium bound form (E1 state) (**23**) (*PDB entry code:1SU4*) since it presents a more open and less structured conformation near the gap corresponding to the insertion at splicing site A in the PMCA pump, facilitating the modelling (**Fig. 3y**)

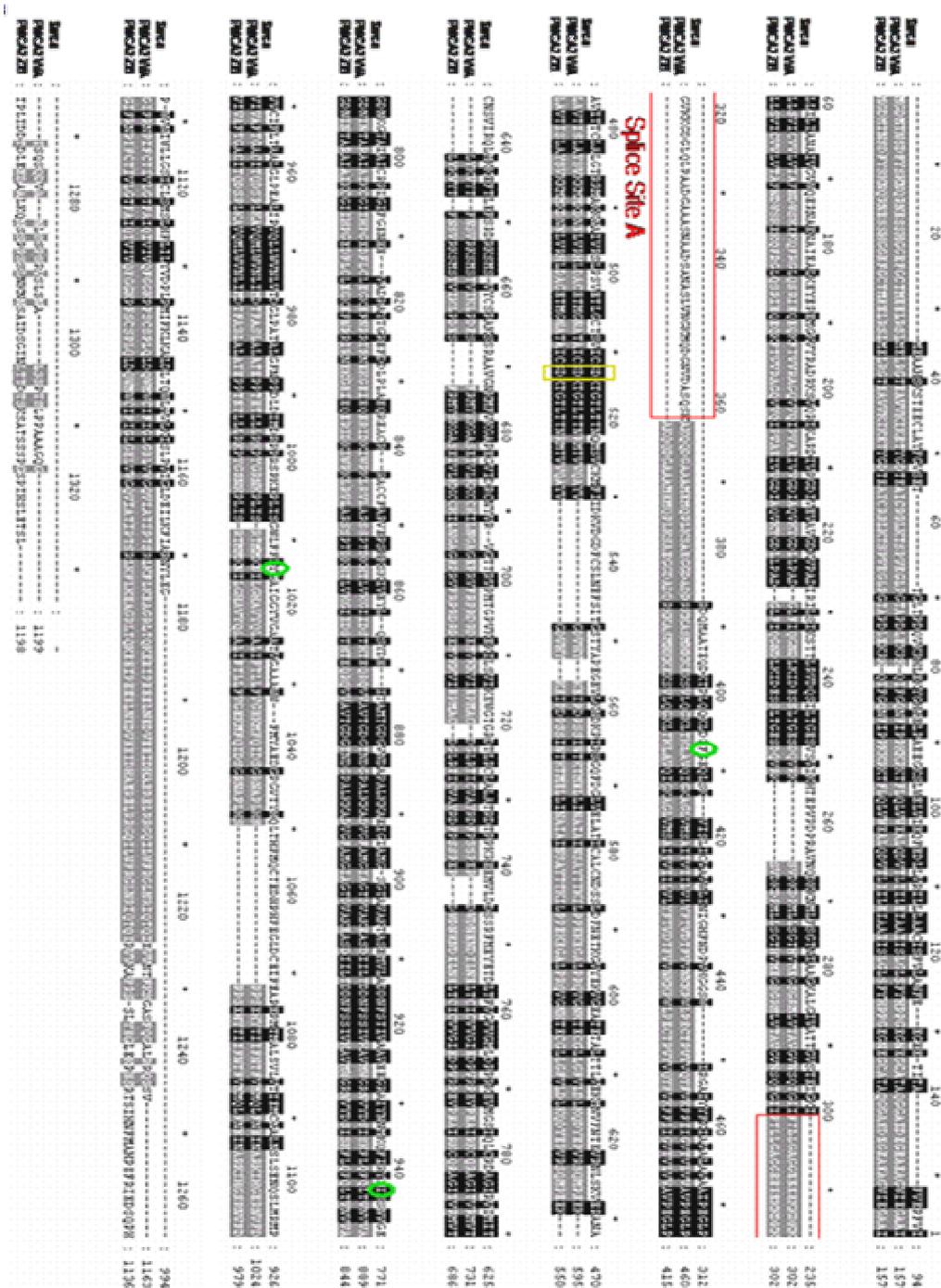


Figure 3x. Sequence Alignment between SERCA and PMCA 2 pumps. Residues are shaded by conservation. Red rectangle indicates the splicing site A insertion. Yellow rectangle indicates the conserved phosphorylation site. Green circles highlight residues involved in TG recognition in SERCA pump not conserved in PMCA pumps. The sequences of the splicing variants *wa* and *zb* are included to illustrate the differences in the insertion length at Site A.

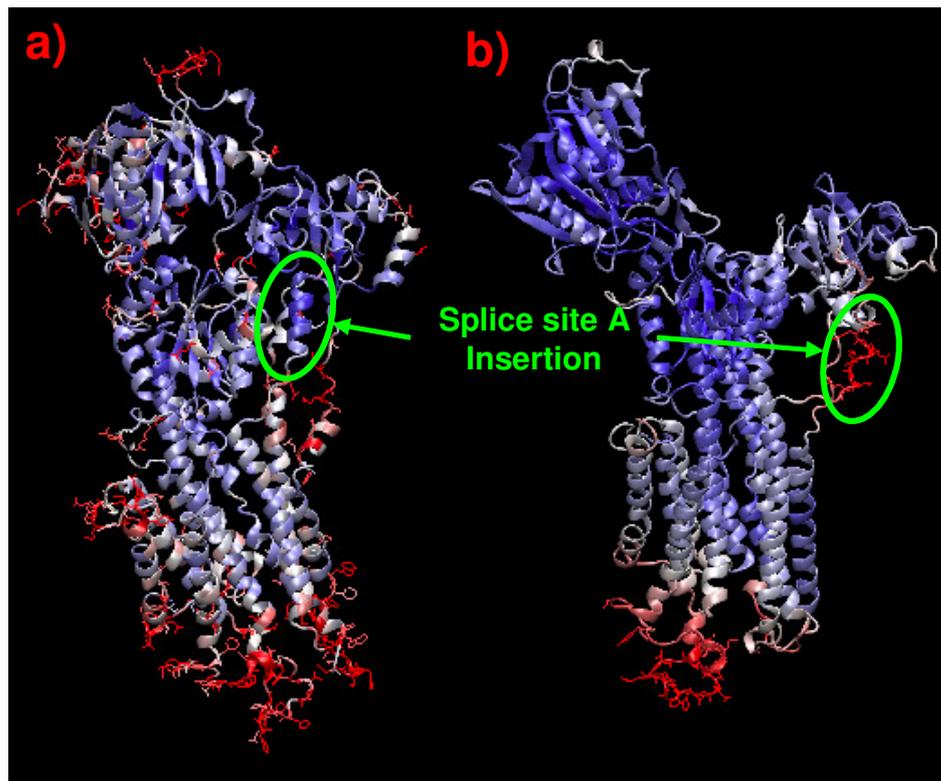


Figure 3y: Choice of the Structural Template. Root mean square deviation fit between SERCA pump in the calcium free E2 (**a**), and calcium bound, E1 states (**b**). Cartoons are colored by B-factor. Blue regions are better defined than red regions. Residues shown in lines display a temperature factor higher than 120 \AA^2 (poor structural determination). Notice the higher exposition of the segment corresponding to the splicing site A in the E1 structure

To gain structural insights into the mechanism of auto inhibition of the PMCA we constructed a 3D model of the *zb* variant of this protein using the above-described alignment. The theoretical model selected superimposed on its structural template with a root mean square deviation of 0,22 nm (**Fig. 3z**). Although the relatively low identity degree (nearly 30%) did not warrant structural reliability up to atomic resolution, the theoretical model revealed some interesting features. The amino acids corresponding to the site of insertion of the splicing site A remain at less than 1 nm from the membrane plane allowing the stretch of residues from KKA to KLA to get in direct contact with the inner leaflet of the plasma membrane. This may suggest that a direct interaction that might pull down the N domain of PMCA hampering its capacity to bind ATP. This block being then released upon addition of acidic phospholipids

Digestion of the peptides resulting from cross-linking experiments have identified peptides DK to VV (region from 206-271 in the first cytoplasmatic loop) and AL to TD

(region from 537 and 544 in the second cytoplasmatic loop) to belong to the binding site of the C-terminal calmodulin binding domain (46). The reactant used had a marked reactivity with sulphur atoms and four sulphur containing residues are indeed found in those peptides (Met257, 265 and 271 in the first segment and Cys571 in the second segment). While, Cys571 is likely to be part of the binding site, the length of the first pinpointed peptide makes it difficult to rationalize this information. Therefore we used the structural information provided by our model to refine this information. Among these residues Met257 resulted buried within the N domain. Furthermore, it is not conserved in isoform 1 of PMCA strongly suggesting that it may not participate of the binding site. Of the remaining two, only Met265 is oriented towards the putative binding site (Fig 3z). Therefore, it can be expected that a mutation of this residue may impair the capability of the pump to bind the calmodulin binding domain.

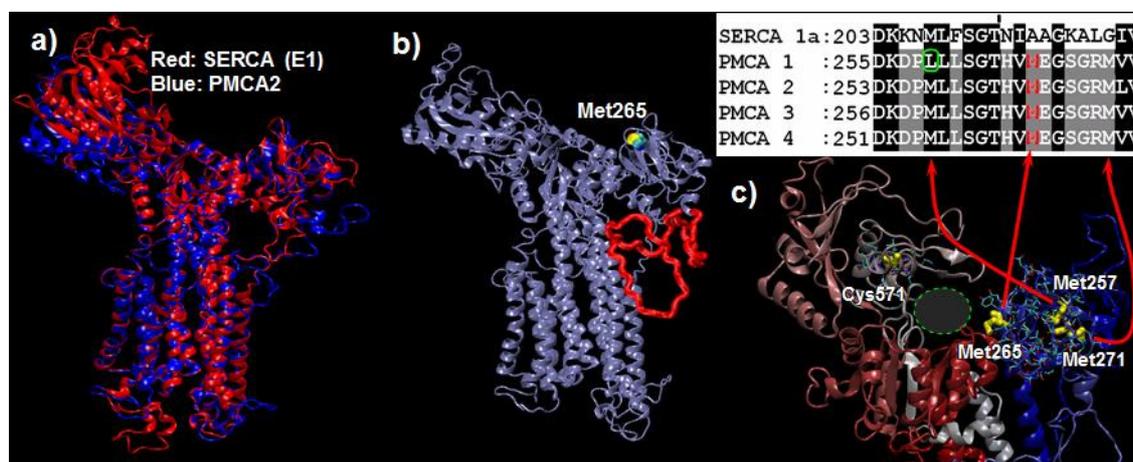


Figure 3z: Structural model of the PMCA2 pump.

a) Superposition of the selected model of PMCA2 on the structure of the SERCA pump in E1 state (PDB entry code 1SU4).

b) Model of PMCA2 pump. The loopy region where splicing inserts occur is marked with red tube and corresponds to the less structurally resolved region of the SERCA pump.

c) The segments near to the auto-inhibitory binding site identified by cross linking experiments are shown in sticks. The four putative sulphur containing residues (Cys571, Met257, Met265 and Met271) within these segments are highlighted in yellow.

Notice that the residue corresponding to Cys571 in the SERCA pump lies within the ATP binding site. Met257 and 271 are not solvent exposed. Furthermore, Met257 is not conserved in PMCA1 as shown in the alignment. The green circle indicates the putative binding site of the calmodulin binding peptide.

To verify this hypothesis, Met265 was mutated to Ala in the PMCA 2 *zb* pump. The mutated PMCA 2 was expressed in parallel with wild type pump in CHO cells. The expression of the PMCA protein in CHO membranes was detected by SDS-PAGE and Western blot (Fig. 3za). Antibody 5F10 recognized a single band at 135 kDa indicating that the full-length polypeptide of the recombinant wild type protein was successfully

expressed. Mutant M265A was expressed at a level similar to that of the wild type protein.

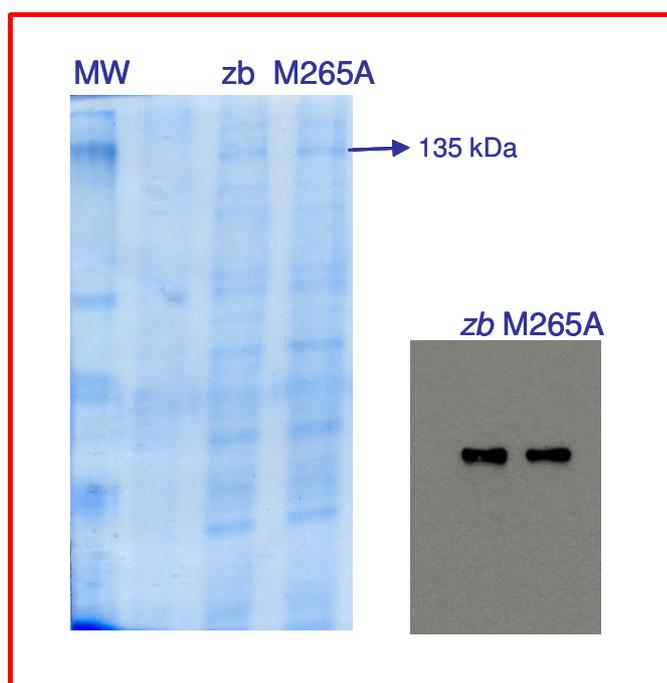


Figure 3za: Expression of PMCA wild type and M265A mutant. 1 μ g of total CHO membranes protein was applied on each lane of an SDS-10% polyacrylamide gel stained with Coomassie Brilliant Blue and subjected to immunoblot analysis with 5F10 monoclonal antibody.

1.1- Functional State of the PMCA 2 zb and M265A mutant.

The activity of the PMCA 2 zb and M265A pumps expressed in transfected CHO cells was evaluated as a function of Ca^{2+} in both the presence and absence of calmodulin. Both conditions, the M265A mutant had a response to calmodulin which was the same as that of the wild type when the concentration of calmodulin was 200 nM. The kinetic parameters of PMCA 2 zb and M265A are showed in **Fig. 3zb**.

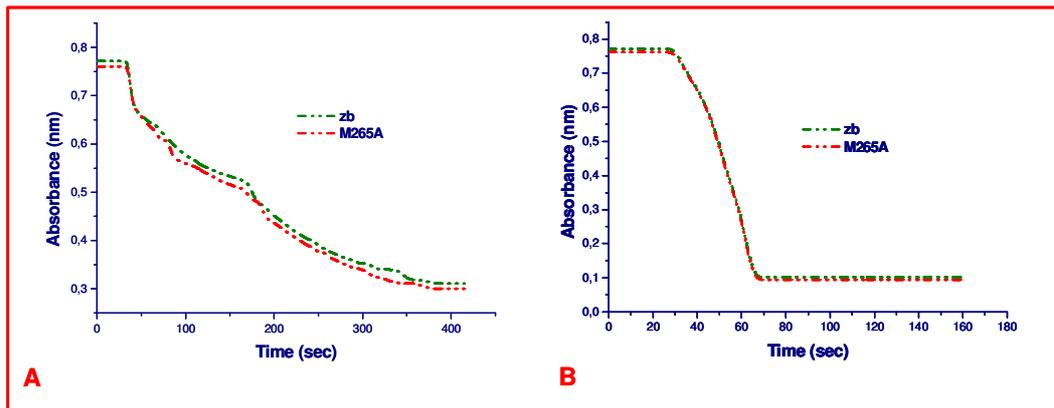


Figure 3zb: The rate of ATP hydrolysis by PMCA 2 *zb* and M265A enzymes. The Ca^{2+} ATPase activity of PMCA membranes was measured at 37 °C as described under "Materials and Methods" in a medium containing 20 mM HEPES, pH 7.00 at 37 °C, 120 mM KCl, 4 mM MgCl_2 , 0.5 mM EGTA, 3 mM , and enough CaCl_2 to give 10 μM Ca^{2+} . **A.** no calmodulin **B.** 200nM calmodulin. This data is a representative curve of 3 experiments with 3 different preparations.

The PMCA 2 *zb* and M265A mutant had the same response to phosphatidyl serine. (**Fig. 3zc**)

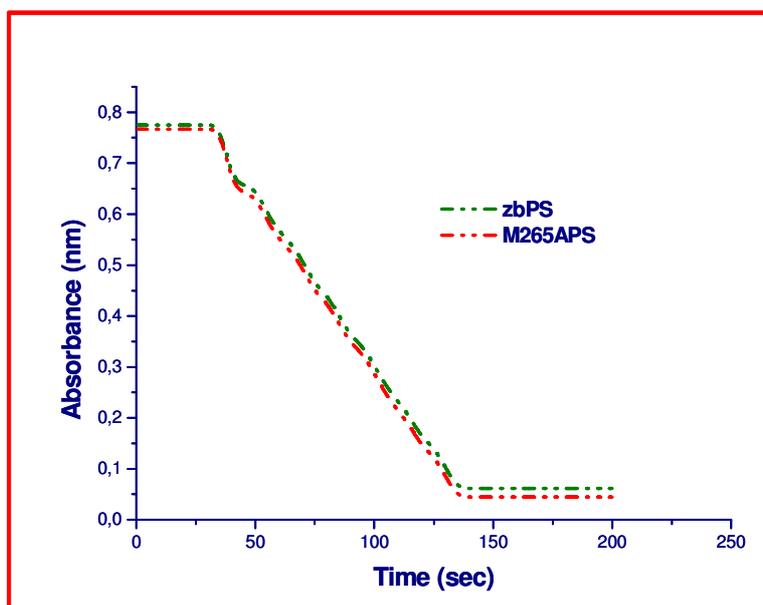


Figure 3zc: Stimulation of the Ca^{2+} ATPase by phosphatidyl serine The Ca^{2+} uptake by microsomal vesicles isolated from CHO cells transfected with PMCA 2 *zb* (green curve), PMCA 2 M265A (orange) are shown. Membrane vesicles were preincubated at 37 °C with 25 μM PS and Ca^{2+} uptake was initiated by the addition of ATP. No differences were found between wild type and mutated pump.

We then tested the effect of the mutation at Met265 on the ability of calmodulin to activate the pump. The Ca^{2+} transport activity was evaluated as a function of the calmodulin concentration. To reach steady state or near steady state conditions, the membranes were preincubated with calmodulin for 5-10 min at 37 °C. The results showed that when the concentration of calmodulin was decreased the activation became higher for M265A with respect to the wild type pump. (**Fig. 3zd and ze**). At 1, 5, 15 nM calmodulin the activity of the mutant was invariably higher than that of the wild type pump. Evidently, the inhibition of Met 265 decreased the affinity of the C-terminal autoinhibitory domain, making it easier for calmodulin to remove it from binding site.

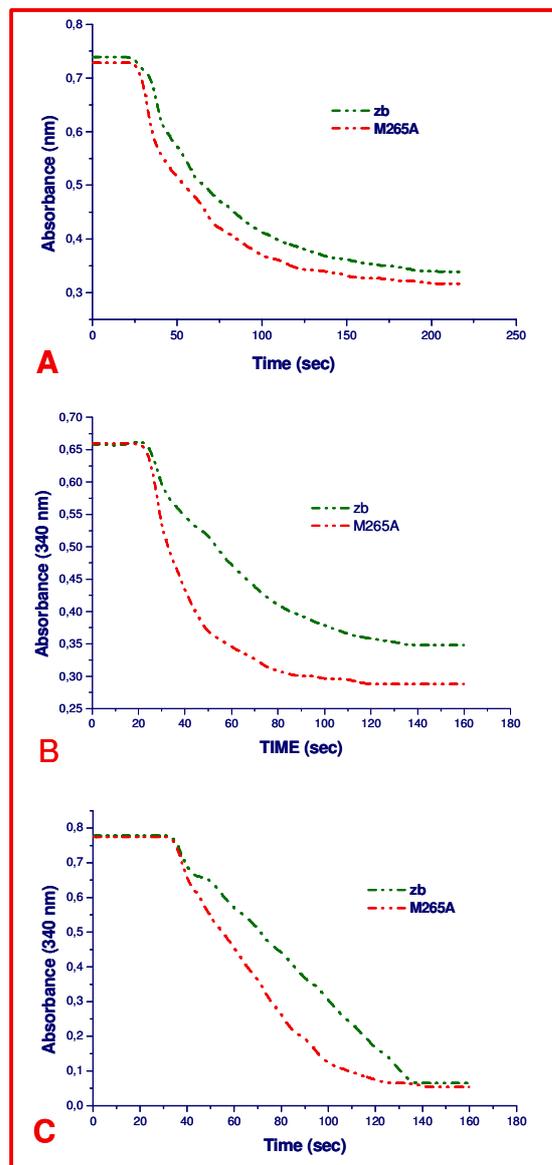


Figure 3zd: Calmodulin dependence of the rate of ATP hydrolysis by the PMCA 2 *zb* and M265A. ATP hydrolysis was measured as described under "Materials and Methods" at a constant free Ca^{2+} of 10 μM . Calmodulin concentrations used were 1, 5 and 15 nM. Three different experiments were performed with different membranes preparations

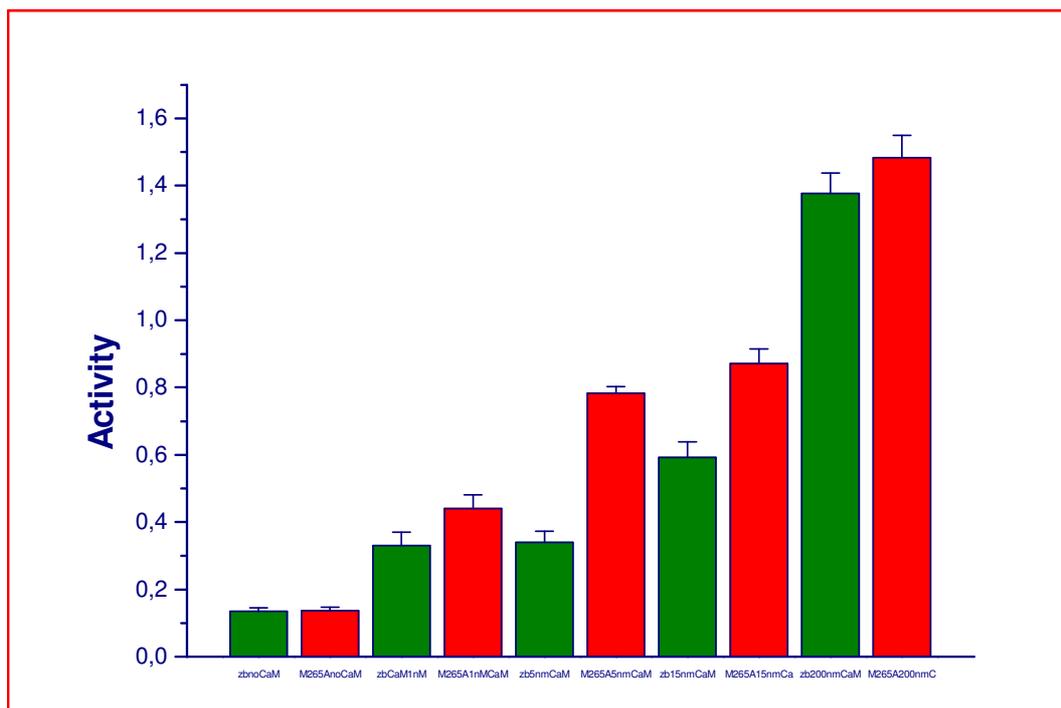


Figure 3 ze: The activity of the PMCA 2 *zb* and M265A at different calmodulin concentrations. The histograms show the means +/- SD of the activity of the pumps. At the 0,05 level the means are significantly different

DISCUSSION

Calcium ions are the main second messengers in all types of eukaryotic cells, in which the free Ca^{2+} concentration in the cytosol is maintained at a very low level (50–150 nM). Fluctuations around this are essential for the control of normal cellular activities and are closely connected with a number of events, e.g., the origin and development of cells, mitotic activity, immune response, muscle contraction, endo- and exocytosis, or modulation of neuronal cells processes also through the control of neurotransmitter release. The precise regulation of the Ca^{2+} homeostasis in cells is a result of the concerted functioning of transporters located in the plasma membrane, systems operating in cell organelles, i.e., the endo/sarcoplasmic reticulum, the mitochondria, the nucleus, and a number of calcium binding proteins. Calcium efflux from excitable cells occurs through two main systems, an electrochemically driven $\text{Na}^+/\text{Ca}^{2+}$ exchanger with low Ca^{2+} affinity, and a plasma membrane Ca^{2+} -ATPase, with much higher Ca^{2+} affinity (Guerini, 1998). The transport capacity of the fully activated $\text{Na}^+/\text{Ca}^{2+}$ exchanger is more than 10 times greater than that of the plasma membrane Ca^{2+} -ATPase. In nonexcitable cells the calcium pump is the sole system responsible for the extrusion of calcium ions given its high Ca^{2+} affinity, the plasma membrane Ca^{2+} -ATPase is considered as the fine tuner of cytosolic calcium ion concentration, and its important role is also reflected in its isoform-specific expression among different cell types.

As found with other ion pumps, PMCAs form a multigene family. The basic plasma membrane calcium pump isoforms are encoded by 4 different genes. Due to alternative mRNA splicing more than 30 variants of the pump exist (Guerini, 1998). Based on functional studies it has been shown that these isoforms and variants differ in regulatory properties.

PMCA isoforms 1 and 4 are, in general, expressed in most tissues, whereas PMCA isoforms 2 and 3 are expressed in a much more restricted manner and, in the adult, predominantly in the brain and striated muscle. Within the brain, PMCA2 is primarily expressed within specialized cell types such as the cerebellar Purkinje cells (Stauffer *et al.*, 1997) and cochlear hair cells (Furuta *et al.*, 1998; Street *et al.*, 1998), where the pump is specifically expressed in the stereocilia of hair cells. Isoform 2 of the pump has properties that distinguish it from other PMCA isoforms: When tested in the cellular environment, it is two to three times more active than the two ubiquitous pumps in

pumping Ca^{2+} out of the cell (Brini *et al.*, 2003). In the isolated state, it shows very high calmodulin affinity (K_d , 2–4 nM) (Elwess *et al.*, 1997); i.e., it becomes fully activated under conditions (e.g., calmodulin and/or Ca^{2+} concentration) that would activate only poorly isoforms 1 and 4.

Alternative splicing is peculiarly complex in PMCA2 because it involves the insertion of up to three novel exons at site A and of two at site C. The A-site insertions are in-frame, creating variant *w* when three exons are inserted (the normal variant without site-A inserts is termed *z*). The insert at site C creates instead a novel stop codon, leading to the truncation of the pump (variants *a* and *b* being the normal full-length pump). The site-C insertions eliminate approximately half of the calmodulin binding domain; those at site A occur next to a domain that binds activatory acidic phospholipids, which, however, also bind to the calmodulin binding domain. The site-A insertion could thus impair the activation by acidic phospholipids, particularly in the C-terminally truncated pump variants, in which the C-terminal phospholipid binding domain also is compromised.

Of interest to the topic of this thesis is how alternative splicing influences the regulation of the PMCAs, primarily their activation by calmodulin. For instance, the stereocilia of the outer hair cells (OHCs) contain a splice variant (*wa*) that has high basal Ca^{2+} ejection activity but fails to respond with a rapid activation to the sudden arrival of a Ca^{2+} load; these properties evidently satisfy the Ca^{2+} homeostasis demands of both the endolymph and of the stereocilia. Even if Ca^{2+} in the endolymph is very low it, is considerably higher than that necessary for the integrity of the tip links. Within the stereocilia, the control of Ca^{2+} is vital to a number of aspects of the mechanotransduction process, e.g., the regulation of adaptation (Gillespie *et al* 2004), the ability to sense the deflection of the ciliary bundle with high sensitivity, and the breaking and regeneration of tip links (Zhao *et al.*, 1996). As mentioned, these peculiar functions of the Ca^{2+} signal have in all likelihood dictated the choice of the PMCA 2 *wa* isoform for the stereocilia. PMCA 2 *zb* has a faster calcium-activation by Ca^{2+} pulse than PMCA 2 *wa*. The lower response of the *wa* variant to large calcium loads makes it unable to eject them rapidly. This may be required to satisfy the calcium homeostasis demands of stereocilia, which contains much less calcium than normal extracellular fluids.

Because resting stereociliary Ca^{2+} is very low, it makes sense to control it with a pump variant that decreases Ca^{2+} to lower concentrations than other isoforms (Elwess *et al.*,

1997), even if it is insensitive to calmodulin. The relative insensitivity of the *wa* pump to calmodulin makes good sense also, given its very high concentration in the stereocilia (Walzer *et al.*, 1996), it would produce permanent maximal activation of pump isoforms normally sensitive to it. On the other hand, any variation in the activity of the pump could conceivably have important consequence on endolymphal Ca^{2+} .

Another important aspect of the PMCA regulation is the activation by acidic phospholipids. Acidic phospholipids, polyphosphoinositides in particular, are the most potent stimulators of the PMCA (Niggli *et al.*, 1981). They reduce the K_m for Ca^{2+} (K_m Ca^{2+}) of the enzyme to 0.3 μM as compared with 0.4 – 0.7 μM in the case of calmodulin stimulation (Penniston *et al.*, 1998). The activation by acidic phospholipids renders the PMCA insensitive to calmodulin activation, i.e., Ca^{2+} -calmodulin and phospholipids are not additive although acidic phospholipids can further reduce the K_m for Ca^{2+} of the calmodulin-stimulated pump (Wang *et al.*, 1991). Data showing that the fully calmodulin-stimulated pump (or of a truncated pump lacking the COOH-terminal autoinhibitory calmodulin binding domain) could be further stimulated by phospholipids indicated that a region(s) other than the COOH-terminal domain must also be involved in the lipid stimulation (Enyedi *et al.*, 1987). This was to be expected, as work with different proteolytic fragments of the PMCA and with synthetic peptides revealed that there are indeed two separate phospholipid binding regions in the pump: one corresponding to the C-terminal calmodulin binding domain and the other situated in the first cytosolic loop immediately preceding the third membrane-spanning domain (Zvaritch *et al.*, 1990). However, the mechanism by which different phospholipids activate the PMCAs is not known. Given the location of the lipid-binding sequences in the pump, one may speculate that the interaction of acidic phospholipids (presumably via their charged headgroups) with the calmodulin binding domain leads to some structural rearrangement that weakens the autoinhibitory intramolecular interactions formed by the C-terminal tail. How phospholipid interactions with the first cytosolic loop further stimulate PMCA activity in a fully calmodulin-activated pump is less obvious. Structural rearrangements of the transduction and catalytic domains could be invoked that may facilitate access of Ca^{2+} to its high-affinity transport sites and/or positively influence a rate-limiting step in the catalytic cycle. Under stimulation by phosphatidyl serine (PS), the PMCA 2 *zb* and *wb*, transfected in CHO cells, have the same response to PS, even if the *wb* variant has an insert close to the end terminal of the phospholipid binding domain (PL-BD), which could be expected, interfere with PL

binding domain nearby. Of the two PL binding domains that in the calmodulin binding domain is apparently more critical in the activation by acidic phospholipids. This is directly supported by the finding that the *wa* variant, which has lost, at least in part, the PL-BD in the CaM binding sequence is totally unresponsive to phospholipids.

A recent finding on PMCA 2 relates its defect to hearing deafness. These uncertainties of mechanism have prompted the experiments described in this thesis:

A variety of recessive mutations of cadherin 23 (*Cdh23*) cause profound deafness and vestibular dysfunction in homozygous mice, (Di Palma *et al.*, 2001) whereas another allele of *Cdh23*, called *ahl*, underlies less severe, age-related hearing loss in many inbred mouse strains (Noben-Trauth *et al.*, 2003). The severity of this age-related hearing loss is significantly increased by heterozygosity for the *dfw2j* deafwaddler allele of *Atp2b2*, which encodes PMCA2, the predominant PMCA of hair bundles. This interaction has been attributed to a reduction in PMCA2 activity that would result in a decrease in extracellular (endolymphal) calcium concentrations around hair bundles, where calcium-dependent, cadherin-mediated adhesion is thought to occur. The 2075G-A mutation is predicted to result in the substitution of methionine for valine at amino acid position 586 (V586M) in the T4–T5 intracellular catalytic loop of PMCA2. The V586M mutation was detected in a mouse, called Tommy, which was affected by sensorineural hearing loss, suggesting that this pump variant may modify the severity of sensorineural hearing loss caused by a variety of factors. The valine residue at position 586 is completely conserved among mouse, rat, and fish PMCA2 orthologues, and either valine or a conservatively substituted residue (isoleucine) is present at this position in all known PMCA1 and PMCA3 aminoacid sequences. When expressed as a recombinant baculovirus protein in Sf9 cells, PMCA2 *wa* V586M has only approximately 50 percent of the calcium ATPase activity of wild-type PMCA 2 *wa*. Other calcium transport activity defect with respect to the wild type *wa* variant have been found in mice, for instance a G283S mutation in deafwaddler mouse and in humans (G293S). The defect is more pronounced in the *dfw* mutant than in the G293S (human) mutant when were analyzed in Baculovirus expression system. The PMCA 2 of the Oblivion mouse (where a Serine is replaced by a Phenylalanine at position 877) is slightly more active than the pumps bearing the 283 and 293 mutations but still much lower that of the wild type *wa*. The Oblivion mutation is localized in the sixth transmembrane domain of the PMCA 2 *wa* pump and the this mice presents ataxia, loss of balance and progressive loss of the auditory function. The study has shown that the

function compromised in the mutated pumps is not the ability to respond to a sudden demand of hyperactivity but the special ability to operate efficiently may be at the nonactivated level.

The autoinhibition of the pump is a very interesting aspect in the regulatory process of the PMCA. The Calmodulin binding to a region in the COOH-terminal portion of the PMCA is located; about 40 residues downstream of the last transmembrane domain (James *et al.*, 1988). In the absence of Ca^{2+} -calmodulin, this sequence acts as an “autoinhibitory” domain; cross-linking studies using labeled peptides have demonstrated that the calmodulin binding domain interacts with two separate regions of the pump, one located in the first cytosolic loop and the other in the major catalytic unit between the phosphorylation and the ATP binding sites (Falchetto *et al.*, 1992). In the absence of calmodulin, the autoinhibitory C-terminal domain is thought to prevent catalytic turnover, keeping the pump in an inactive state. An elevation in the cytoplasmic Ca^{2+} activates the calmodulin, which then interacts with high affinity with its binding sequence bound the autoinhibitory domain of the PMCA, thereby releasing it and the inhibition. This regulatory mechanism is similar to that of other Ca^{2+} - calmodulin-dependent enzymes such as smooth muscle myosin light-chain kinase or Ca^{2+} /calmodulin-dependent protein kinase I.

To investigate whether Calmodulin indeed removes its binding domain from the cytosolic loops of the pump, we have introduced a mutation in one of the two sites that interact with the CaM binding domain in the cytosolic loops of the pump. Prior to this, the PMCA pump was modeled to decide the residue that should have been mutated. Previous sequencing work on the peptides resulting from cross-linking experiments have identified peptides DK to VV (region from 206-271 in the first cytoplasmatic loop) and AL to TD (region from 537 and 544 in the second cytoplasmatic loop) as belonging to the binding site for the C-terminal calmodulin binding domain (Falchetto *et al.*, 1992). The reactant used in that work had a marked reactivity with sulphur atoms and four sulphur containing residues are indeed found in those peptides (Met257, 265 and 271 in the first segment and Cys571 in the second segment). While, Cys571 is likely to be part of the binding site, the length of the first pinpointed peptide makes it difficult to rationalize this information. Among these residues Met257 resulted buried within the N domain. Furthermore, it is not conserved in isoform 1 of PMCA strongly suggesting that it may not participate of the binding site. Of the remaining two, only Met265 is oriented towards the putative binding site. This amino acid was mutated to Alanine (M265A).

The activity of the PMCA 2 zb and M265A pumps expressed in transfected CHO cells evaluated as a function of Ca^{2+} in both the presence and absence of calmodulin shows that the M265A mutant has a response to calmodulin which was the same as that of the wild type at saturating concentrations of calmodulin. The activation by CaM was higher in the M265A pump with respect to wild type pump at 1nM, 5nM and 15 nM calmodulin concentrations. This suggests that the autoinhibitory sequence was bound with less affinity to the intramolecular binding site in the Met265 mutant as less calmodulin was required to relieve the inhibition. Evidently, the inhibition of Met 265 decreased the affinity of the C-terminal autoinhibitory domain, making it easier for calmodulin to remove it from binding site.

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