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A NEW CONFORMATION IN SERCA AND PMCA Ca^{2+} PUMPS REVEALED BY A PHOTOACTIVATABLE PHOSPHOLIPIDIC PROBE

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The purpose of this work was to obtain structural information about conformational changes in the membrane region of the sarcoplasmic reticulum (SERCA) and plasma membrane (PMCA) Ca^{2+} pumps. We have assessed changes in the overall exposure of these proteins to surrounding lipids by quantifying the extent of protein labeling by a photoactivatable phosphatidylcholine analog [^{125}I]TID-PC/16 under different conditions. We determined that: (1) Incorporation of [^{125}I]TID-PC/16 to SERCA decreases 25% when labeling is performed in the presence of Ca^{2+}. This decrease in labeling matches qualitatively the decrease in transmembrane surface exposed to the solvent calculated from crystallographic data for SERCA structures. (2) Labeling of PMCA incubated with Ca^{2+} and calmodulin decreases by approximately the same amount. However, incubation with Ca^{2+} alone increases labeling by more than 50%. Addition of C28, a peptide which prevents activation of PMCA by calmodulin yields similar results. C28 has also been shown to inhibit ATPase SERCA activity. Interestingly, incubation of SERCA with C28 also increases [^{125}I]TID-PC/16 incorporation to the protein. These results suggest that in both proteins there are 2 different E_1 conformations: one that is auto-inhibited and is in contact with a higher amount of lipids (Ca^{2+}+C28 for SERCA, Ca^{2+} alone for PMCA) and one in which the enzyme is fully active (Ca^{2+} for SERCA and Ca^{2+}-calmodulin for PMCA) and that exhibits a more compact transmembrane arrangement. These results are the first evidence that there is an autoinhibited conformation in these P-type ATPases, which involves both the cytoplasmic regions and the transmembrane segments.

Although membrane proteins constitute more than 20% of the total proteins, the structure of only few of them is known in detail. An important group of integral membrane proteins are ion-motive ATPases. These proteins belong to the family of P-type ATPases, which share in common the formation of an acid-stable phosphorylated intermediate as part of its reaction cycle. Crystallographic information is available for a few members of this family. There are several crystal structures of the Ca^{2+} pump of sarcoplasmic reticulum (SERCA) revealing different conformations (1-5), and recently, crystal structures of the H^+-ATPase (6) and of the Na,K-ATPase were reported as well (7)

We are interested in obtaining structural information about the plasma membrane calcium pump (PMCA). This pump is an integral part of the Ca^{2+} signaling mechanism (8). It is highly regulated by calmodulin, which activates this protein by binding to an auto-inhibitory region and changing the conformation of the pump from an inhibited state to an activated one (8,9). Crystallization of PMCA is particularly challenging because there is no natural source from which this protein can be obtained in large quantities. Besides, the presence of several isoforms in the same tissue further complicates efforts to obtain a homogeneous sample suitable for crystallization.

Information about the structure and assembly of the transmembrane domain of an integral membrane protein can also be obtained from the analysis of the lipid–protein interactions. In this work, we have used a hydrophobic photolabeling

method to study the non-covalent interactions between PMCA and surrounding phospholipids under different experimental conditions that lead to known conformations. We employed the photoactivatable phosphatidylcholine analog 1-palmitoyl-2-[9-[2′-[^{125}I]iodo-4′-(trifluoromethyldiazirinyl)-benzyloxycarbonyl]-nonaoyl]-sn-glycero-3-phosphocholine [^{125}I]TID-PC/16 that has been previously used to analyze lipid–protein interfaces (10-12) . This reagent locates in the phospholipidic milieu and upon photolysis it reacts indiscriminately with its molecular neighbors. It is thus possible to directly analyze the interaction between a membrane protein and lipids belonging to its immediate environment (13-15). By measuring the amount of labeling of SERCA in conditions that promote conformations for which there are well resolved crystal structures, we were able to validate this photolabeling approach as a convenient tool for analyzing conformational changes within transmembrane regions. Furthermore, using this technique on PMCA and comparing the results obtained for SERCA we were able to draw structural conclusions about these proteins under activated and inhibited states.

Experimental Procedures

Reagents. All chemicals used in this work were of analytical grade and purchased mostly from Sigma Chemical Co. (USA). Recently drawn human blood for the isolation of PMCA was obtained from the Hematology Section of the Hospital de Clínicas General San Martín and from Fundación Fundosol, (Argentina). Blood donation in Argentina is voluntary and therefore the donor should provide informed consent to the donation of blood and the subsequent legitimate use of the blood by the transfusion service.

Purification of PMCA from human erythrocytes. PMCA was isolated from calmodulin depleted erythrocyte membranes by the calmodulin-affinity chromatography procedure (16). Briefly, membrane proteins were solubilized in a 0.5% $C_{12}E_{10}$ containing buffer. After centrifugation supernatant was loaded into a Sepharose-calmodulin column in the presence of 1mM Ca^{2+}. The column was thoroughly washed with 0.05% $C_{12}E_{10}$ containing buffer. PMCA was eluted in 20% (w/v) glycerol, 0.005% $C_{12}E_{10}$, 120 mM KCl, 1 mM $MgCl_2$, 10 mM MOPS-K, pH 7.4 at 4 ºC, 2 mM EGTA, 2 mM DTT and stored in the same buffer after the addition of 2 mM $CaCl_2$. It has been previously demonstrated that both the conformation and the activity of the protein are preserved in either solubilized or reconstituted purified preparation compared with that located in the erythrocyte (16). Protein concentration after purification was about 10 µg/ml. No phospholipids were added at any step along the purification procedure. By measuring inorganic phosphate (see *Phospholipid quantification*) we estimated that less than 1 mol of natural phospholipids per mol of PMCA is present in the purified enzyme. The purification procedure described preserves transport activity and maintains the kinetic properties and regulatory characteristics of the enzyme in its native milieu (16).

SERCA preparation. SERCA was directly solubilized with $C_{12}E_{10}$ (0.5%) from sarcoplasmic reticulum membranes (prepared from rabbit skeletal muscle as previously described (17)). This sample was diluted 100 times in a medium containing 20% (w/v) glycerol, 120 mM KCl, 1 mM $MgCl_2$, 10 mM MOPS-K, pH 7.4 at 4ºC, 2 mM EGTA, 2 mM $CaCl_2$. Protein concentration after dilution was 10 µg/ml.

Measurement of Ca^{2+}-ATPase activity. Ca^{2+}-ATPase activity was measured at 37°C as the initial velocity of release of Pi from ATP as described previously (16). The incubation medium was 7 nM PMCA, 120 mM KCl, 30 mM MOPS, 3.75 mM $MgCl_2$, 1 mM EGTA, 1.1 mM $CaCl_2$, 140 µM soybean phospholipids, 800 µM $C_{12}E_{10}$, and 2 mM ATP (pH 7.4 and $[Ca^{2+}]$ = 140 µM). Release of Pi was estimated according to the procedure of Fiske and Subbarow (18). Measurements were performed in a *Jasco V-630 Bio* spectrophotometer.

Preparation of [^{125}I] TID-PC/16. TTD-PC/16 (tin precursor) was a kind gift of Dr. J. Brunner (ETHZentrum, Zuerich, Switzerland). [^{125}I]TID-PC/16 was prepared by radioiodination of its tin precursor according to Weber and Brunner (12). After the reaction was completed, the mixture was extracted with chloroform/methanol (2:1, v/v) and [^{125}I]TID-PC/16 was purified by passage through a silica gel column (2.5 ml) using chloroform:methanol: water:acetic acid (65:25:4:1, v/v) as solvent. The elution was monitored by TLC/autoradiography and the fractions containing

the product were dried and stored at -20ºC.

Phospholipid quantification. Phospholipid concentration was measured according to Chen et al. (19) with some modifications. Samples and standards containing 10–100 nmol phosphorus were dried by heating at 100ºC. Mineralization was carried out by adding 0.1 ml HNO_3, 0.9 ml $HClO_4$ and incubating at 190ºC for 30 min. Inorganic phosphate was determined after Fiske and Subbarow (18).

Labeling procedure. A dried film of the photoactivatable reagent was suspended in DMPC/$C_{12}E_{10}$ mixed micelles containing 10 µg/ml of the membrane protein. The samples were incubated for 20 min at 37ºC before being irradiated for 15 min with light from a filtered UV source ($\lambda \approx 360$nm).

Radioactivity and protein determination. Electrophoresis was performed according to the Tris–tricine SDS–PAGE method (20). Polypeptides were stained with Coomassie BlueR, the isolated bands were excised from the gel, and the incorporation of radioactivity was directly measured on a gamma counter. The amount of protein was quantified by eluting each stained band, as previously described (21) including Serum albumin in each gel as a standard for protein quantification. Specific incorporation was calculated as the ratio between measured radioactivity and amount of protein determined for each band.

Proteolysis of PMCA. Proteolysis of PMCA was performed for 3 minutes, in the presence of Tris-HCl 25mM, pH 7.4 at 37ºC, 100 µM free Ca^{2+} and 0,22 µg/ml of TLCK-treated chymotrypsin (stored as a stock in dimethylformamide). The reaction was stopped by a tenfold excess of ovomucoid trypsin inhibitor solution at 4ºC.

Immunoblotting. The slabs obtained after SDS-PAGE were electroblotted onto a poly (vinylidene difluoride) microporous membrane (Immobilon) by means of a semidry electroblotting system as described by Lauriere (22). Before immunostaining, the Immobilon sheets were treated with blocking solution and then incubated with appropriate dilutions of the primary antibodies (see below) in PBS at room temperature for 1 hr. After rinsing with PBS, the streptavidin-phosphatase conjugate anti-mouse and –rabbit IgG (Sigma, St Louis, MO) was added for 1 hr; this step was followed by rinsing. Color development was obtained using 3 amino-9-ethylcarbazole (Sigma).

Antibodies. The monoclonal antibodies used here were raised against different regions of human erythrocyte Ca^{2+}-pump, *i.e*. 5F10 recognizes the central region (residues 719-738), JA9 the N-terminus (residues 51-75) and JA3 the C-terminus (residues 1156-1180). The numbering of residues corresponds to human PMCA4b. Specificity was demonstrated by their reaction with the purified enzyme in an ELISA system and by their staining in a Western blot (23-25)

Analysis of SERCA structure and Accesible Surface Area. The crystal structures used for comparison were: 1iwo.pdb (8), 1su4.pdb (7) and 2ear.pdb (6). The transmembrane regions were taken as explicitly defined in Uniprot for Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 Accession number P20647. The ASA of the transmembrane helices was calculated with MolMol (26, 27) with a solvent radio of 1.4 Å. Superposition of crystal structures was performed with POSA (28).

Analysis of the Data. All measurements were performed by triplicate to quintuplicate unless is specified in the figures. SDS gels presented in Results were chosen as representative of at least three independent experiments.

RESULTS

The probe. [^{125}I]TID-PC/16 was previously used to identify and characterize regions within membrane proteins that interact with lipids (12,13). This reagent is a PC analogue endowed with a photoactivatable group at the end of one of the fatty acyl chains (Fig.1). Its physicochemical behavior is indistinguishable from that of PC, *i.e.*, it shows identical mobility on TLC plates using different solvent systems (data not shown). Moreover, its interaction with transmembrane regions has been shown to be identical to that of PC (14,15). Additionally, regarding membrane protein-lipid interactions PC is considered the least selective lipid (29). In other word, transmembrane regions are expected to behave as an homogeneous surface displaying no specific binding sites for this phospholipid. This fact simplifies the interpretation of our results, allowing for a direct correlation between level of [^{125}I]TID-PC/16 incorporation and amount of protein surface

exposed to surrounding lipids.

EXPERIMENTS WITH SERCA

We have chosen SERCA as the P-type ATPase paradigm because a well-defined three dimensional structure is available for most of its conformations (1-5). Analysis of the results will be based on the SERCA reaction cycle proposed by de Meis and Vianna (1979) (30), as shown in Fig. 2. The cycle starts with binding of Ca^{2+} to E_1. E_1Ca is represented by the structure known as 1su4 (2). E_1Ca binds ATP. E_1Ca-ATP is represented by the 1vfp (4) structure. The release of ADP leads to E_1PCa. The structure of E_1PCa is known as 3ba6 (5). Conformational transition and release of 2 Ca^{2+} ions to the lumen yields E_2P, represented by the 3b9b (5) structure. Finally, E_2 conformation (stabilized by the inhibitor thapsigargin) is represented by structures 2ear (1) and 1iwo (3). The most striking rearrangement in protein structure occurs when the enzyme binds Ca^{2+}, *i.e.* between 2ear (E_2TG) and 1su4 (E_1Ca). The different assembly of transmembrane segments in these conformations results in a decrease of the protein average exposure to surrounding lipids whenever Ca^{2+} is present. The extent of interaction between SERCA and membrane lipids under each condition was quantified by calculating the accessible surface area (ASA) of the transmembrane region. The results thus obtained are shown in table 1.

Effect of Ca^{2+}. Fig. 3 shows the specific incorporation of [^{125}I]TID-PC to SERCA in the presence of EGTA, Ca^{2+} and, EGTA and thapsigargin. The extent of protein labeling measured in the presence of calcium is lower than that found in the presence of EGTA and considerably lower than that measured in the presence of EGTA and thapsigargin. This experimental result is in qualitative agreement with the predicted lipid-exposed area of the protein under each condition based on ASA calculations (Table 1). The effect of thapsigargin is probably the result of the stabilization of the enzyme in E_2, *i.e.* under this condition SERCA is quantitatively fixed in a unique conformation. Indeed, it has only been possible to obtain SERCA crystals in the presence of EGTA provided that thapsigargin is also present.

EXPERIMENTS WITH PMCA

Effect of Ca^{2+} and calmodulin. Since experiments with SERCA show that the incorporation of [^{125}I]TID-PC/16 yields the results expected according to ASA calculations, we decided to quantify the amount of labeling of PMCA (for which no crystal structures are yet available) in different conditions. Figure 4 shows that [^{125}I]TID-PC/16 binds to PMCA in different amounts according to the conformational state of the enzyme. In the presence of Ca^{2+}, the incorporation of [^{125}I]TID-PC/16 is nearly 50% higher than the incorporation of the reagent in the absence of this ion. This result is opposite to that obtained for SERCA under identical conditions and indicates that in PMCA, the amount of area directly exposed to membrane lipids increases upon Ca^{2+} binding.

The effect of 1.6 μM calmodulin on the incorporation of [^{125}I]TID-PC/16 to PMCA is also shown. Labeling in the presence of calmodulin was carried out either in the presence of EGTA or in the presence of 100 μM Ca^{2+}. The amount of labeling of PMCA in the presence of EGTA and calmodulin is identical to that obtained in the presence of EGTA alone. Under this condition in which no binding of calmodulin to PMCA occurs, a possible sequestering effect of the reagent by calmodulin is tested. Binding of calmodulin to the enzyme dramatically reduces the extent of protein labeling to about 52% of the labeling in the presence of Ca^{2+} alone, indicating that in this condition PMCA exhibits less lipid exposure than in any other conformational state. This behavior is similar to that observed for SERCA after addition of Ca^{2+} which leads E_2 to E_1. This suggests that regarding the transmembrane domain, the PMCA conformation in which the enzyme is no longer auto-inhibited *i.e.,* in the presence of calmodulin and Ca^{2+}, is similar to that adopted by SERCA after addition of Ca^{2+} alone.

Ca^{2+} concentration used in these labeling experiments was similar to that routinely used for Ca^{2+} ATPase measurements. Experiments performed under the same conditions but lowering Ca^{2+} concentration to 1 μM yielded identical results to those shown in Fig. 4. Furthermore, Mg^{2+} failed to mimic the effects observed with Ca^{2+}, *i.e.* protein labeling in the presence of 3.75 mM Mg^{2+} was indistinguishable from that determined in the presence of EGTA. This evidence strongly support the specificity of the effects exerted by Ca^{2+} on the extent of protein

labeling.
In addition, Fig.4 shows the effect of removal of PMCA C-terminus by proteolysis with TLCK-chymotrypsin. It is well known that proteolysis of PMCA by chymotrypsin yields a pump that is fully active lacking the C-terminal region, and hence, does not bind calmodulin. The inset shows the SDS-PAGE of the experiment. An immunoblot of the truncated PMCA (not shown) reveals that JA3, a monoclonal antibody raised against residues 1156-1180, did not react with the truncated protein indicating the complete removal of the C-terminus. In the presence of EGTA the level of reagent incorporation to truncated PMCA is indistinguishable from that obtained under the same condition for the undigested enzyme. On the other hand, Ca^{2+} decreased the incorporation of [^{125}I]TID-PC/16 in the truncated PMCA to 83%. This result indicates that cleavage of the calmodulin binding region of PMCA mimics not only its activation by calmodulin but also the effect of calmodulin on the incorporation of [^{125}I]TID-PC/16 in its transmembrane domain.

Effect of C28. In order to further explore the structural relationship between level of exposure to phospholipids and activation by calmodulin, we assayed the effect of C28, a peptide made after the sequence of the calmodulin binding domain of PMCA. Fig. 5 shows that, as previously reported (31) C28 inhibits with high affinity the calmodulin activation of PMCA. This behavior is interpreted as the consequence of C28 binding to calmodulin which preserves an auto-inhibited conformation of PMCA. In such condition, C28 also reverts the effect of calmodulin on [^{125}I]TID-PC/16 incorporation to PMCA. It has also been reported that at micromolar concentrations, C28 partially inhibits the Ca^{2+}-ATPase activity of SERCA (31). As shown in Fig. 5, the presence of C28 results not only in the described inhibitory effect on SERCA activity but also in an almost 20% increment of [^{125}I]TID-PC/16 incorporation. This result suggests that indeed C28 is able to promote an inhibited conformation in SERCA analogous to the one observed in PMCA in the absence of calmodulin.

COMPARISON BETWEEN SERCA AND PMCA
The effect of other ligands. Table 2 summarizes the relative incorporation of [^{125}I]TID-PC/16 in the presence of different ligands, both in SERCA and in PMCA.
From the observation of this table we can draw the following conclusions: 1) Ca^{2+} has opposite effects on SERCA and PMCA when compared to pumps that had been labeled in the presence of EGTA or EGTA and thapsigargin. In SERCA Ca^{2+} decreases the amount of labeling, while in the case of PMCA it increases it. 2) Addition of calmodulin and Ca^{2+} (CaM + Ca^{2+}) to PMCA decreases the amount of labeling in similar manner that Ca^{2+} does to SERCA. 3) Addition of C28 to SERCA produces a larger level of incorporation of [^{125}I]TID-PC/16, in a similar way to the effect of Ca^{2+} on PMCA in the absence of calmodulin. 4) Addition of La^{3+} and Ca^{2+} increases the labeling as Ca^{2+} alone does 5) Finally, addition of La^{3+} + Ca^{2+} and ATP, a condition which blocks the interconversion between E_1P and E_2P (32), yields the same amount of labeling as Ca^{2+} alone. This suggests that E_1P, the conformation stabilized by La^{3+} and ATP, has similar exposition to phospholipids than E_1CaI. La^{3+} alone was not tested in SERCA.
Finally, vanadate has no effect on the incorporation if [^{125}I]TID-PC/16 in the absence of Ca^{2+}, and in the presence of Ca^{2+} has opposite effects in SERCA and PMCA. In SERCA, it increases the level of incorporation when compared to Ca^{2+} alone, and in the case of PMCA it decreases the incorporation level, again, when compared to Ca^{2+} alone. These results are consistent with the idea that vanadate partially antagonize the effects of Ca^{2+} (33,34)

DISCUSSION

We have developed a methodological approach based on the use of a photoactivatable probe to study the structure of the transmembrane region of PMCA in different conformations. By quantifying the amount of labeling by [^{125}I]TID-PC/16 we were able to estimate the differential interaction of PMCA conformers with surrounding

phospholipids. The procedure is validated by making comparisons with the known crystal structures of SERCA, for which a well-defined three dimensional structure is available (1-5).

A major structural difference between SERCA and PMCA is the presence of a C-terminal auto-inhibitory region in the latter. Activation of PMCA by calmodulin has been explained by binding of this protein to the C-terminal end, thus removing the inhibitory interactions from the cytosolic core. It should be noted that this hypothesis for auto-inhibition does not predict changes in the transmembrane region.

However, the results in this paper show for the first time that the auto-inhibited conformation is a distinct one (at least in the E_1Ca phase of the PMCA reaction cycle) and that the conformational changes induced by auto-inhibition do expose additional surfaces to phospholipids. Because the vast majority of phospholipids present in our experiments is phosphatidylcholine and the photoactivatable reagent used is a phosphatidylcholine analogue, no higher affinity sites are expected to be sensed under our experimental conditions. Nevertheless, the possibility that upon Ca^{2+} binding, sites with different affinity for phospholipids are either exposed or buried cannot be ruled out. Efforts are currently underway in our laboratory in order to explore this alternative. It is worth noting that although PMCA has specific sites for acidic phospholipids (35), no such sites have been identified for neutral phospholipids.

The auto-inhibited conformation was also obtained by adding the calmodulin-binding peptide C28 to an E_1Ca-CaM state of PMCA, showing the reversibility of the conformational transition (36).

Calmodulin binding peptides from related transporters have shown to inhibit SERCA as well (11). We show that addition of C28 in conditions in which this peptide inhibits SERCA also induces an increase in [^{125}I]TID-PC/16 labeling in this pump as well. This fact suggests that C28 can induce an inhibitory conformation alike to the one present in PMCA in the absence of calmodulin. This evidence allows us to propose the existence of two different E_1 conformations: E_1I being the auto-inhibited PMCA and a E_1A the activated one (in the presence of calmodulin or after removing the C-terminal tail by controlled proteolysis). In this sense, the E_1A is the conformation naturally present in SERCA. Addition of C28 to SERCA mimics the E_1I conformation present in the auto-inhibited PMCA. It is noteworthy that a short hydrophilic peptide like C28 drives a profound change at the hydrophobic transmembrane region.

It is interesting to analyze the effects of two inhibitors of PMCA, La^{3+} and vanadate. La^{3+} is known to stabilize E_1Ca, and in the presence of ATP blocks the reaction cycle in E_1P (32). The fact that La^{3+} and ATP produces the same level of incorporation than Ca^{2+} alone suggests that the transmembrane arrangement of E_1P is similar to the one of E_1Ca (E_1I). On the other hand, early experiments on the kinetics of vanadate inhibition showed that it is antagonized by Ca^{2+}(33,34) a result consistent with the idea of Ca^{2+} and vanadate binding to alternate conformations. In addition, vanadate binds to the E_2 conformation of SERCA, and thus the apparent vanadate affinity also depends upon the E_1 to E_2 equilibrium. The intermediate level of reagent incorporation measured in the presence of vanadate and Ca^{2+} is indeed indicative of the coexistence of E_1 and E_2 states.

In conclusion, by detecting differences in the access of the photoactivatable probe [^{125}I]TID-PC/16 to both PMCA and SERCA we were able to assess lipid exposure of the transmembrane domain of well characterized kinetic intermediates. Moreover, we were able to uncover the involvement of the transmembrane region in the auto-inhibition of PMCA. Remarkably, we found out a similar conformational state generated by C28 in SERCA.

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FOOTNOTES

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The Abbreviations used are: PMCA, Plasma Membrane Calcium Pump; SERCA Sarcoplasmic Reticulum Calcium Pump; EGTA, Ethylene Glycol Tetraacetic Acid; ASA: Accesible Surface Area; [^{125}I]TID-PC/16, 1-O-hexadecanoyl-2-O-[9-[[[2-[^{125}I]iodo-4-(trifluoromethyl-3H- diazirin-3-yl)benzyl]oxy]carbonyl] nonanoyl]-sn-glycero-3-phosphocholine; TLC, Thin Layer Chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; DTT, Dithiothreitol; DMPC, (1,2-dimyristoyl- sn -glycero-3-phosphocholine) ; $C_{12}E_{10}$, Polyoxyethylene(10)dodecyl ether / 3,6,9,12,15,18,24,27,30-decaoxadotetracontan-1-ol; Tris, tris(hydroxymethyl) aminomethane; PBS, Phosphate Buffered Saline

LEGEND TO FIGURES

Fig. 1. Photoactivatable phosphatidylcholine analog 1-palmitoyl-2-[9-[2′-[^{125}I] iodo-4′-(trifluoromethyldiazirinyl)-benzyloxycarbonyl]-nonaoyl]-sn-glycero-3-phosphocholine, [^{125}I]TID-PC/16.

Fig. 2. SERCA reaction cycle proposed by De Meis and Vianna (38) with the pdb names of the matching crystallized structures of the main reaction steps 2ear (6); 1iwo(8); 1su4 (7); 1vfp (9); 3ba6 (10); 3b9b (10).

Fig. 3. Relative specific incorporation of [125I]TID-PC to SERCA in the presence of 1mM EGTA and 300 nM thapsigargin and in the presence of 100 μM Ca^{2+}. The incorporation of [^{125}I]TID-PC to SERCA in the presence of EGTA alone was taken as 100% (control). The inset shows a typical SDS-PAGE of SERCA. Values shown are the mean ± SEM of 6 independent experiments.

Fig. 4. Effect of Ca^{2+}, calmodulin and partial proteolysis on PMCA specific incorporation of TID-PC. PMCA was labeled in the presence of 1 mM EGTA and 100 μM Ca^{2+}. Reagent incorporation was tested under these same conditions adding 1.6 μM CaM to the incubation medium or using a partially digested enzyme which lacks the CaM binding domain. Values are referred to that obtained for [^{125}I]TID-PC incorporation of PMCA in the presence of EGTA alone which was taken as 100% (control) The inset shows the SDS-PAGE of the remaining undigested enzyme (138 kDa) and the resulting fragment (120 kDa) after partial proteolysis with TLCK-chymotrypsin. Values shown are the mean ± SEM of at least 3 independent experiments

Fig. 5. Effect of C28 on [^{125}I]TID-PC incorporation. PMCA and SERCA were labeled in the presence of 1 μM and 20 μM C28 respectively. These concentrations of C28 effectively inhibit CaM activation of PMCA and ATPase activity of SERCA as shown in the right panel. Values shown are the mean ± SEM of at least 3 independent experiments.

Table 1: Accesible surface area of SERCA transmembrane region .

Crystal structure	Condition	ASA (Å^2)	Relative hydrophobicity (%)	Conformation
2ear	Tg + EGTA	8701	100	E_1
1iwo	Tg + EGTA	8235	94.6	E_1
3b9b	BeF^{4-}	8164	93.8	E_1
1su4	Ca^{2+}	7114	81.7	E_2
1vfp	Ca^{2+} + AMPPCP	7295	83.8	E_2
3ba6	E_1PCa	7350	84.5	E_2

Crystallographic data used for ASA calculations (5) were taken from references 6 (2ear); 8 (1iwo); 7 (1su4); 9 (1vfp); 10 (3ba6 and 3b9b).

Table 2: RELATIVE INCORPORATION OF [^{125}I]TID-PC/16

	SERCA		PMCA	
Condition	Conformation	Specific incorporation	Conformation	Specific incorporation
EGTA	E_2	100 ± 3	E_2	100 ± 3
EGTA + TG	E_2	151 ± 10	E_2	100 ± 3
EGTA + CaM	E_2	ND	E_2	99 ± 3
EGTA + C28	E_2	98 ± 3	E_2	99 ± 3
EGTA + Vanadate	E_2	97± 5	E_2	100 ± 7
Ca^{2+}	E_1	75 ± 4	E_1I	155 ± 2
Ca^{2+} + CaM	E_1	70 ± 7	E_1A	80 ± 2
Ca^{2+}+ CaM + C28	E_1I	ND	E_1I	144 ± 3
Ca^{2+} +C28	E_1I	117 ± 3	E_1I	150 ± 9
Ca^{2+}+TG	E_1; E_2	91 ± 3	E_1I	154 ± 8
Ca^{2+}+ La^{III}	E_1	ND	E_1I	156 ± 4
Ca^{2+} + ATP + La^{III}	E_1P	89 ± 4	E_1P	155 ± 8
Ca^{2+} +Vanadate	E_1; E_2	92 ± 3	E_1; E_2	125 ± 9

Results are the mean ± SEM of 3 to 7 independent experiments. ND: not determined. E_1A: an activated state of E_1; E_1I: an inhibited state of E_1.

Figure I

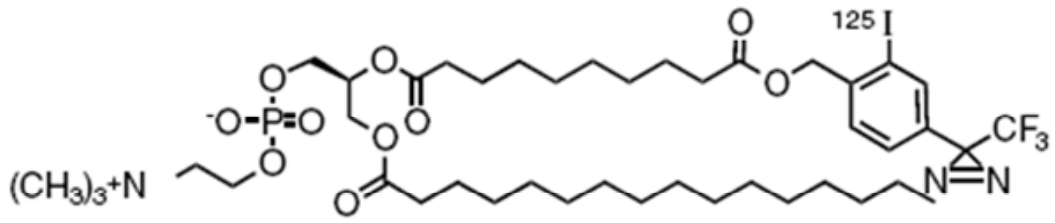


Figure 2

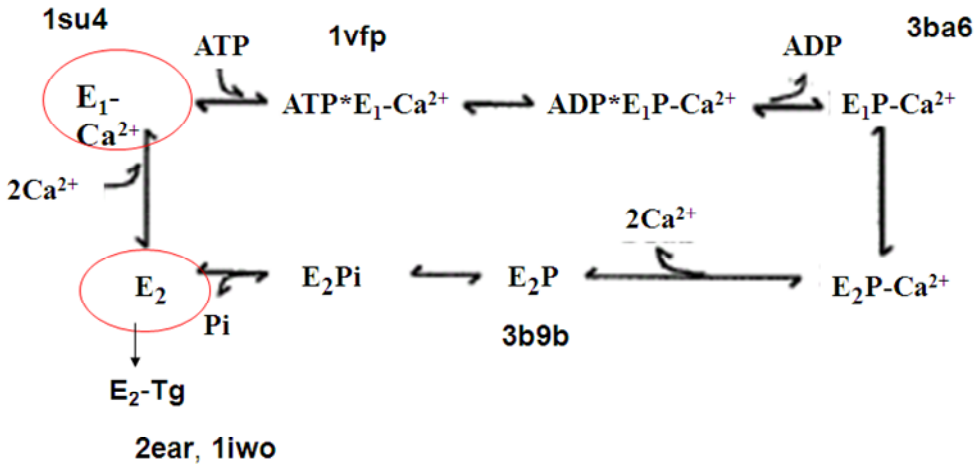


Figure 3

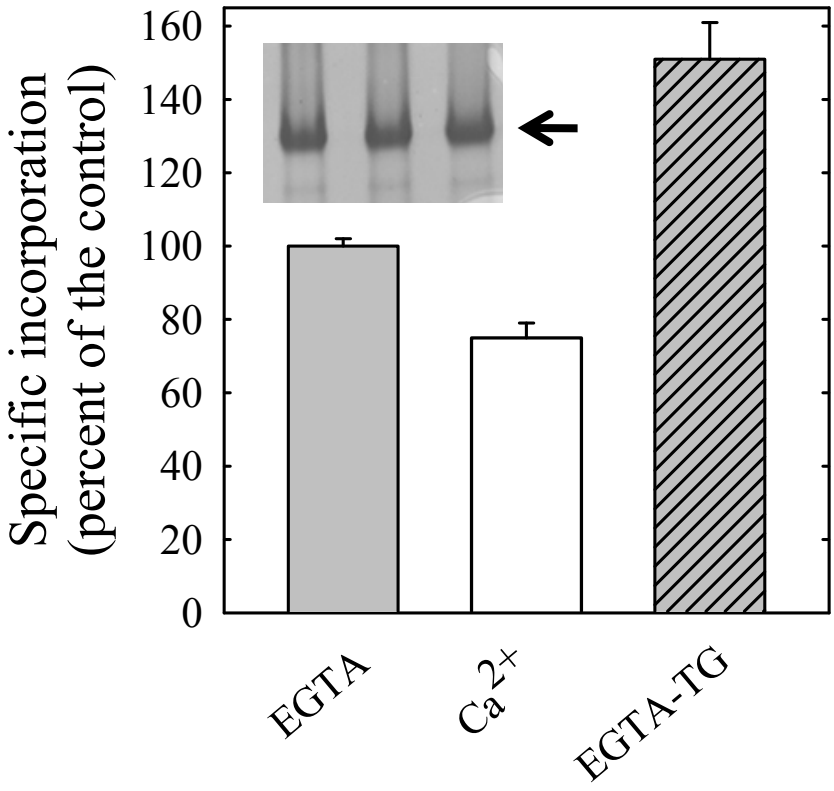


Figure 4

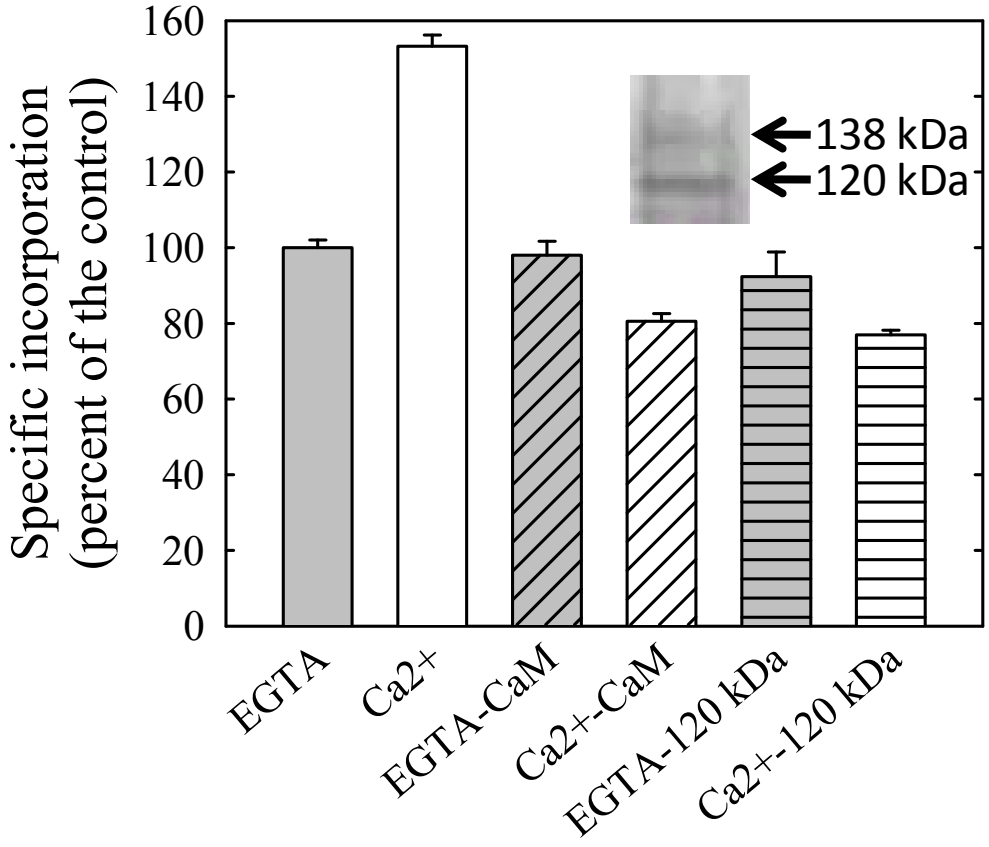
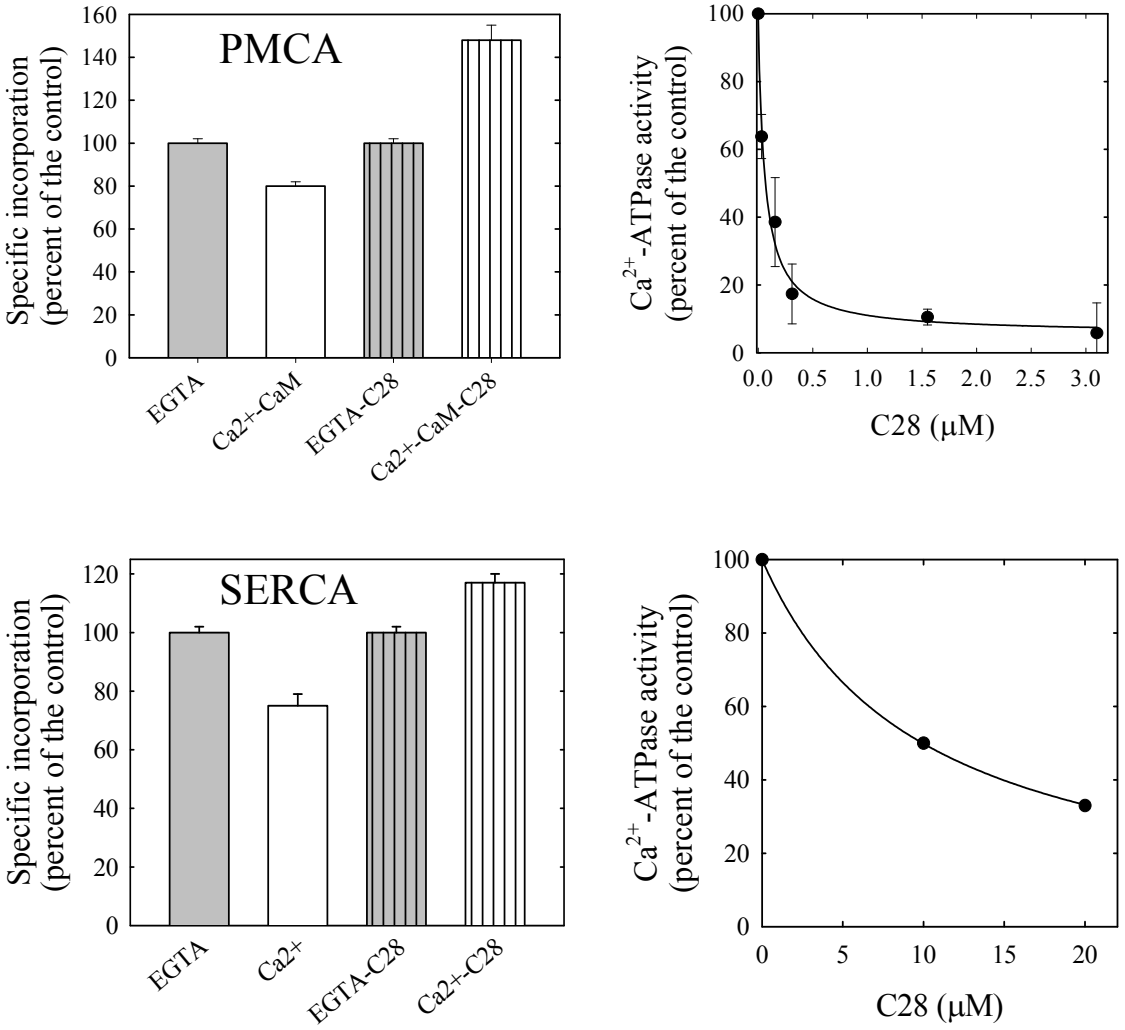


Figure 5



A new conformation in serca and PMCA Ca^{2+} pumps revealed by a photoactivatable phospholipidic probe

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